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PURIFICATION AND CHARACTERIZATION OF A NEW FUNGAL EXTRACELLULAR ENZYME

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PROČIŠĆAVANJE I KARAKTERIZACIJA NOVOG FUNGALOG EKSTRACELULARNOG ENZIMA

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Sažetak: Enzim celobioza dehidrogenaza iz *Corynascus thermophilus (CtCDH) je* eksprimiran u *Aspergillus niger*. Najbolji transformant je odabran za provjeru proizvodnje enzima, te za proizvodnju u Erlenmajerovim tikvicama. Ispitivanja tijekom proizvodnje pokazala su sličnost s divljim tipom *Ct*CDH dok je *Ct*CDH eksprimiranim u kvascu *Pichia pastoris* pokazao nižu specifičnu aktivnost.. Enzim je pročišćen u dva stupnja. Molekularna masa iznosi 90 kDa. Kinetičke konstante enzima *Ct*CDH su više u usporedbi s vrijednostima objavljenim za divlji tip *Ct*CDH kao i za *Ct*CDH exprimiran u *Pichia pastoris*. Enzimu CDH je također testirana sposobnost *in situ* proizvodnje H_2O_2 u reakciji s enzimom nespecifična peroksigenaza kako bi se uspostavio ekološki prihvatljiv proces oksidacije etilbenzena. U tu svrhu su testirani različiti CDH varijanti iz *Myriococcum thermophilum* sa povećanim afinitetom prema kisiku. Varijant N748G/ T750Q se pokazao kao najučinkovitiji. Ograničavajući faktor u primjeni ovakve vrste je stabilnost enzima, na što se buduća istraživanja moraju orijentirati.

Ključne riječi: celobioza dehidrogenaza, *Aspergillus niger*, karakterizacija, primjena CDH, ekološki prihvatljiv proces oksidacije etilbenzena

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PURIFICATION AND CHARACTERIZATION OF A NEW FUNGAL EXTRACELLULAR ENZYME

Tanja Lešić, 676/BPI

Abstract: Cellobiose dehydrogenase from *Corynascus thermophilus* (*Ct*CDH) was cloned into *Aspergillus niger* for heterologous expression. The best transformant selected for screening was also selected for the enzyme production in Erlenmeyer flasks. During the production, the recombinant *Ct*CDH showed similar characteristics to the wild-type *Ct*CDH, whereas *Ct*CDH produced in *Pichia pastoris* shows a lower specific activity. The expressed enzyme was purified in two-step procedure. The molecular mass was ~90 kDa. Kinetic constants of *Ct*CDH were higher compared to the wild-type *Ct*CDH and the one expressed in *Pichia pastoris.* CDH was also tested for its ability of the *in situ* production of H_2O_2 and it was combined with unspecific peroxygenase to establish an eco-friendly process for ethylbenzene oxidation. For that purpose different CDH variants from *Myriococcum thermophilum* with increased oxygen reactivity have been tested. The variant N748G/ T750Q was found to be most efficient. A limiting factor in application of this type seems to be the enzyme's stability on which further research must focus
Keywords: cellobiose dehydrogenase, *Aspergillus niger*, characterization, CDH application, eco-friendly process of ethylbenzene oxidation.

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1. INTRODUCTION

Lignocellulose-based biomass, although complexed and degradation-reluctant, is also the most abundant renewable polymer on the Earth and as such of tremendous interest for development of sustainable and environmental friendly bioprocesses (Langston et al, 2011; Borisova et al., 2015; Hemsworth et al., 2015). Fungi and bacteria are the main actors in degradation of such robust material since they secrete a various number of lignocellulose-degrading enzymes (Tan et al., 2015). Among those enzymes is cellobiose dehydrogenase, an extracellular flavocytochrome, produced by white and brown rot fungi as well as phytopathogenic and saprotrophic fungi (Ludwig et al., 2010, Harreither et al., 2011). Catalytic properties of CDHs depend on their origin, although they share a common bipartite structure containing b-type cytochrome domain connected to a flavodehydrogenase domain via flexible linker (Hallberg et al., 2002; Harreither et al., 2012).

As a major fraction of the lignocellulose-degrading group of enzymes, CDH is often secreted by fungi in sufficient amounts, but cultivation of its natural producers, as well as protein isolation and purification are difficult and time consuming. Another approach is to clone *cdh* genes in different well-known expression hosts which enable faster, more reliable and more efficient enzyme production as well as the possibility of genetic manipulations (Ludwig et al., 2013). Thus, the first aim of this work was the transformation of the gene for *Corynascus thermophilus* CDH in *Aspergillus niger*, as well as to express, purify and characterize the enzyme.

Since CDHs exhibit some unique catalytic properties, a number of significant applications have been introduced. Analytical CDH-based biosensors, different assays for qualitative and quantitative analyses of substrates, usage in biodegradation processes etc. are just few examples. In this work we have also proposed a new interesting application of different CDH variants in organic industry as a part of cascade reaction. This reaction is bioconversion of ethylbenzene to phenylethanol, and beside CDH, another enzyme, unspecific peroxygenase (UPO) is crucial for this reaction. Thus, we made an effort to establish efficient two-phase bioconversion of ethylbenzene to phenylethanol using UPO and CDH and to characterize these enzymes.

2. THEORETICAL PART

2.1. LIGNOCELULLOSIC BIOMASS

The most abundant raw material on the Earth, lignocellulosic biomass, is also the most attractive in terms of development of different ecologically and economically sustainable bioprocesses (Nigam and Pandey, 2009; Philippoussis, 2009; Langston et al., 2011). The main reasons for such great interest worldwide are its renewability and abundance as well as the fact it does not compromise food or feed production (Monlau et al., 2013).

Two main components of lignocellulosic biomass, cellulose (40 - 50%) and hemicellulose (25 - 35%) are associated together and embedded in the third component, lignin (15 – 20 %) (Hallberg et al., 2002; Baček et al., 2012). Due to different ecological and genetic factors these percentages differ from one to another raw material (Sluiter et al., 2010).

Cellulose is a linear polymer of D-glucopyranose linked by β-1,4-glycosidic bonds. Hydroxyl groups interact with each other, forming hydrogen bonds which enable connecting cellulose chains into a crystal structure (Palonen, 2004; Zamocky et al., 2006; Turner et al., 2007).

The chemical composition and structural properties of heteropolysaccharides, known as hemicelluloses, depend on plant species, type of plant tissue and its developmental stage. Hemicelluloses differ by the main sugar residue in the backbone, such as most common xylans and mannans, and other like glucans. Xylan is predominant form of hemicellulose in most plants. Its backbone is formed of β-(1,4)-linked D-xylopyranose monomers while different groups can be present in the side chains (Wyman et al., 2005; Zamocky et al., 2006; Turner et al., 2007).

In general, lignin is composed of a large group of different aromatic polymers. Specific alcohols like coniferyl-, sinapyl- and p-coumaryl alcohols are precursors of lignin biosynthesis. Lignin is one of the most degradation-challenging materials on the Earth and has a plant protective role. As such it is the most limiting factor in lignocellulose biomass utilization (Henriksson et al., 2000a; Vanholme, 2010).

2.2. FUNGI AND THEIR ROLE IN DEGRADATION OF LIGNOCELLULOSE

Fungi together with bacteria play a great role in carbon cycle, by recycling and reusing carbon and in that way making life on Earth sustainable (Langston et al, 2011; Tan et al., 2015). Making a large taxonomic group, fungi display great phenotypic diversity as a reflection of different environment adaptions. As a result of these variations, which enable them growth on lignocellulosic material, specific lignocellulose-degrading enzymes are secreted (Kracher, 2016).

Important enzymes producers are brown and white rot fungi, as well as composting and plant pathogenic fungi from two most significant phyla Basidiomycota and Ascomycota (Zamocky et al., 2006; Ludwig et al, 2010). Besides the stated ones, several anaerobic species from ruminant gastrointestinal tracts are capable of cellulose degradation (Dashtban et al., 2009).

Common activities of different enzymes lead to lignin degradation. Being unable to penetrate into lignocellulosic matter, peroxidases act by generating molecular radicals which cause lignin oxidation. Other efficient enzymes are laccases which include redox mediators that are able to diffuse through inner structure to nonphenolic compounds, causing their oxidation (Henriksson et al., 2000a; Zamocky et al., 2006; Kracher, 2016).

Since the composition of hemicelluloses varies among different plants, different enzymes are necessary for hydrolysis of these complexed structures. Among these enzymes are xylanases which hydrolyze xylan into oligomers and β-xylosidase which continues degradation of oligomers into xylose. Also β-mannanases, arabinofuranosidases and α-L-arabinanases take part in hemicellulose-degrading process, depending whether the composition of hemicellulose is mannan-based or arabinofuranosyl-containing (Dashtban et al., 2009).

For years, efficient degradation of cellulose had been associated to hydrolytic enzymes; however, synergy of oxidative enzymes that enhances the cellulose degradation was recently introduced (Borisova et al., 2015; Tan et al., 2015). During the hydrolytic degradation process chain-cleaving endocellulases (endoglucanases) create chain breaks providing many reaction points for exocellulases' (cellobiohydrolases) activity. Cellobiose is then released as the main product and gets converted to glucose monomers by β-glucosidase (Zamocky et al., 2006; Turner et al., 2007; Langston et al., 2011; Morgenstern et al., 2014; Schulz, 2015; Tan et al., 2015; Kracher, 2016). Recently, the role of oxidative enzymes in the

biomass deconstruction has been recognized. Cellobiose can also be oxidized to cellobionolactone by cellobiose dehydrogenase. During this process two electrons are obtained which are further transferred to lytic polysaccharide monooxygenase (LPMO). LPMOs are then able to activate molecular oxygen and oxygenate cellulose, leaving chain breaks. In that way these enzymes boost activity of well-known cellulases which contributes to generally more efficient cellulose degradation (Morgenstern et al., 2014; Borisova et al., 2015; Tan et al., 2015; Schulz, 2015; Kracher, 2016).

2.3. CELLOBIOSE DEHYDROGENASE

The fungal flavocytochrome enzyme cellobiose dehydrogenase (CDH; EC 1.1.99.18) is an extracellular oxidoreductase produced by wood-degrading basidiomycetes and ascomycetes as well as phytopathogenic species (Ludwig et al., 2010; Kracher et al., 2015). Its secretion by various different fungi, under cellulolytic conditions, indicates its important role in wood decomposing process (Henriksson et al., 2000b; Hallberg et al., 2002; Pricelius et al., 2009; Ludwig et al., 2010; Harreither et al., 2011; Ludwig et al., 2013). Although the catalytic properties of CDHs depend on their origin, the common feature of enzymes belonging to CDH family is the structure. They are usually produced as monomers with two domains, the dehydrogenase domain (DH) with nonconvalently bonded FAD and the cytochrome domain (CYT) which contains haem *b* as a prosthetic group. A flexible, papain sensitive, 20-30 residues long linker connects these domains (Tasca et al., 2011; Harreiether, 2012; Ludwig et al., 2013). Depending on the degree of glycosylation, the molecular mass of the enzyme is within the range from 85 up to 101 kDa (Zamocky et al., 2006; Ludwig et al., 2013).

The catalytic reaction takes place at the larger flavodehydrogenase domain; a carbohydrate is oxidized to corresponding lactone wherein FAD is reduced to $FADH_2$. The smaller cytochrome domain behaves as built-in mediator, as it transfers electrons to its redox partner LPMO. This process is believed to clarify the *in vivo* function of CDH. Another possible way of $FADH_2$ reoxidation is the electron transfer to different one- or two-electron acceptors such as iron ions, quinones etc. (Ludwig et al., 2010, Ludwig et al., 2013, Felice et al., 2013; Tan et al., 2015).

2.3.1. Structure

With their complex designed structure, CDHs are unique as extracellular enzymes (Kracher et al., 2015). The crystallization of full-length CDHs remained unsuccessful for a long time. Just recently, Tan et al. have achieved to crystallize full length CDHs from *Myriococcum thermophilum* and *Neurospora crassa*. Until then crystal structure of only proteolytically separated domains from *Phanerochaete chrysosporium* CDH have been known (Tan et al., 2015; Kracher, 2016). Catalytic properties of both separated dehydrogenase domain and full-length CDH have been studied in this thesis.

The reported CYT crystal structure from *Phanerochaete chrysosporium* exhibits resemblance to the fold of the antibody Fab V_H domain. The 190 residues-long fragment has a β-sandwich fold with an average diameter of 35 Å, made by two antiparallel, five and six-stranded β-sheets (Rotsaert et al., 2003; Zamocky et al., 2006; Ludwig et al., 2010; Ludwig et al., 2013; Schulz, 2015). On the surface of the domain there is a hydrophobic binding site for heam *b*, which is hexacoordinated by histidine and a methionine. The redox potential of haem *b* in CYT is relatively low (100-160 mV vs.SHE; pH 7.0) as it is believed to be a consequence of specific Met/His ligation (Zamocky et al., 2006; Ludwig et al., 2013). That ligation is very uncommon among heam proteins, but it is well conserved in CDH's CYT domain regardless of CDH's origin. This fact indicates high importance in extracellular electron transfer leading to a greater understanding of the *in vivo* function of CDH (Hallberg et al., 2002; Ludwig et al., 2010; Ludwig et al., 2013; Kracher , 2016).

Formerly, the dehydrogenase domain of CDH is believed to be a separate enzyme. Forms of CDH without cytochrome domain had previously been found, probably as a result of fungal proteases activity (Zamocky et al., 2006; Ludwig et al., 2010). This was supported by the fact that the oxidation of an electron donor takes place at DH fragment where at the same time FAD is reduced to $FADH_2$ and can be reoxidized without cytochrome domain (Henriksson et al.2000b; Hallberg et al.,2002; Zamocky et al., 2006).

In 2002 Hallberg et al. determined the crystal structure of DH domain from *Phanerochaete chrysosporium.* Based on the sequence similarity DH belongs to a large family of glucose-methanol choline (GMC) oxidoreductases. The peanut-shaped, 540-residue large DH domain has a *p*-hydroxybenzoate hydroxylase (PHBH)-like fold. The structure reveals a flavin-binding subdomain of α/β type and a substrate-binding subdomain as seven-stranded

β-sheets with six helices. The FAD-binding domain features so called Rossman-fold motif, characteristic for FAD or NAD-dependent proteins. The 335 residues-large substrate-binding subdomain and flavin-binding domain at their interference form a funnel-shaped tunnel, which leads to well buried active site (Hallberg et al, 2002; Zamocky et al., 2006; Schulz, 2015).

The DH domain of some ascomycete CDHs also contain a separate carbohydrate-binding module (CBM) connected the DH (Schulz, 2015).

The full-length crystal structures of two CDHs reported by Tan et al. played a major role in efforts to understand interactions between CYT and DH domain. They reported a shielded inter-domain electron transfer (IET) from DH domain to the CYT in CDH from *Myriococcum thermophilum*. Crucial interactions for efficient IET are those of heam *b*'s propionate A in CYT with the DH active site. The way the CYT and DH domains are arranged is a closed state with an edge-to-edge distance of 9 Å. The distance fits within the indispensable 14 Å limit for an efficient IET. A channel leading from the protein surface to the active site provides an opportunity for the substrate to enter and the product to exit the active site, even while CDH remains in the closed IET-competent state (Figure 1). Besides this closed state, *Mt*CDH may be present in different conformational states, such as the, so called, open state since there is no domain' association. This state is necessary for CDH's *in vivo* function as it allows the interaction between the CYT domain and LPMO, the final electron acceptor (Tan et al., 2015; Schulz, 2015). The *in vivo* function will be further discussed in the following chapters.

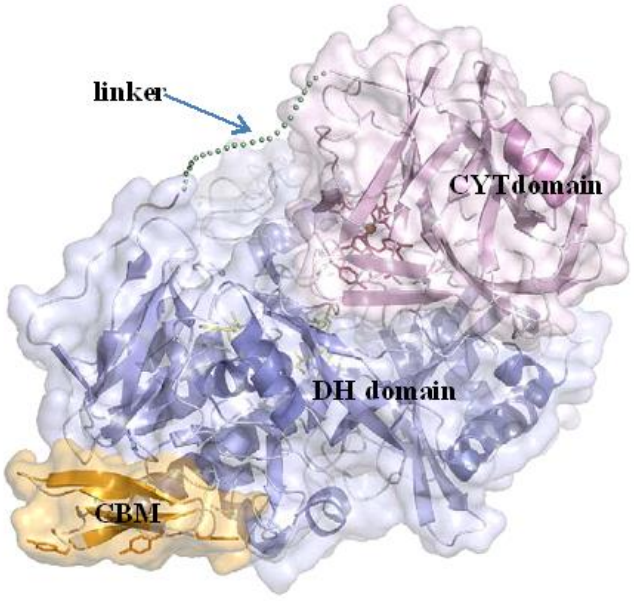

Figure 1. *Mt*CDH in the closed state shown as a ribbon drawing (adapted from Tan et al., 2015).

2.3.2. Classification of CDH

Based on phylogenetic analyses of all known *cdh* genes, the heterogeneous CDH enzyme family is separated into several branches. The branch of the class I CDH represents basidiomycetous CDHs and single copies of *cdh* genes in their genomes are discovered. Class II consists of more complex gene sequences of ascomycetous origin with up to three copies in their genome. A common ancestor of these two phyla probably existed formerly, since among all CDHs, there is a high perseverance of CYT sequence with characteristic His/Met iron coordination. Class II is further divided into two subclasses IIA and IIB according to the fact whether CDH contains a type one carbohydrate-binding module, approximately 30 residue-long (CBM) or not (Schulz, 2015).

An existence of a third class of CDHs was proposed in 2008 but to this day no CDH from this class has been characterized (Harreither et al., 2011; Ludwig et al., 2013; Kracher , 2016). The classification of the CDH family enzymes can be seen in Figure 2.

An attempt of heterologous expression of CDH III from *Aspergillus oryzae* in *Aspergillus niger* has also been a part of this thesis.

The CDH origin defines its characteristics such as sequence length, substrate specificity, pH and temperature optima etc. (Schulz, 2015). In general, CDHs of basidiomycetous origin display optimum activity in mesophilic conditions and mostly acidic pH values. Ascomycetous CDHs are highly active within different temperature extremes and pH range. These characteristics are probably consequences of the adaption to the natural habitats of fungi (Harreither et al, 2011).

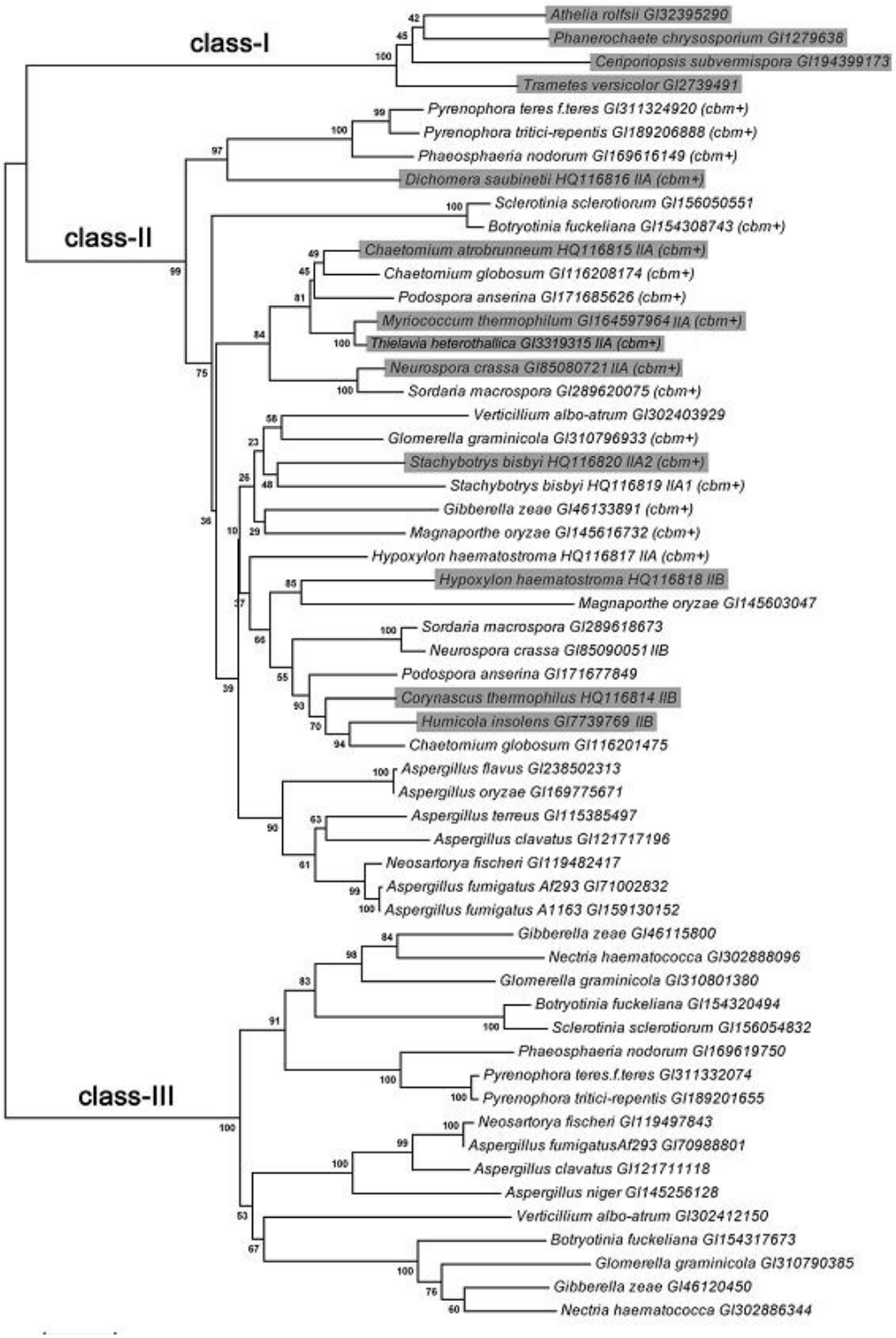

Figure 2. Phylogenetic tree of 56 CDH protein sequences. Basidiomycetous sequences are in class I, and ascomycetous CDHs are divided into class II and class III. So far isolated CDHs are marked in gray shading (adapted from Harreither et al, 2011).

As has already been stated, the substrate spectrum of CDHs depends on their origin**.** β-1,4 interlinked saccharides are preferable substrates for all CDHs. CDH displays highest catalytic efficiencies for β-D-cellobiose. Comparing kinetic constants for cellobiose between class I and class II CDHs, turnover numbers (k_{cat}, s^{-1}) appear to be higher for class I CDHs but the affinity for cellobiose is lower (high K_M values). Although ascomycetous CDHs are less discriminating against lactose, maltose and glucose in contrast to class I, the catalytic efficiency is not as high as for the natural substrate cellobiose (Schulz, 2015; Kracher, 2016).

Several amino acids in the substrate-binding subdomain, such as the conserved Glu in class I CDHs and Asn in class II CDHs at position 279, seem to play a major role in substrate specificity. Furthermore, class I CDHs have a very specific cellulose-binding site at the DH domain. None of class II CDHs has that site, but one subclass has a carbohydrate-binding module. Lacking this module, CDHs from the other subclass are not able to bind to cellulose, but they demonstrate a broad substrate spectrum (Harreither et al., 2011).

Different pH profiles between basidiomycetous and ascomycetous CDHs are a reflection of the adaption to different environment. Basidiomycetes seemed to be adjusted to acidic environmental conditions since secreted CDHs showed pH optima between pH 3.5 and 4.5. Based on their pH profiles with cyt *c* assay, class II CDHs are divided into three groups, acidic, intermediate and neutral/alkaline. A pH optimum about pH 5.0 and tight pH profile are characteristic for the first group. Although members of the second group have also acidic pH optima, they display broad pH working range as they keep high activity at pH 7.5. The last group exhibit pH optima in neutral or alkaline pH area. Some CDHs, such the one from *Corynascus thermophilus*, have different pH optimum depending on activity assay used. Thus pH optimum for the DCIP assay is at 5.0, while for the cyt *c* is at pH 7.5 (Harreither et al., 2011; Schulz, 2015).

2.3.2. Electron transfer

2.3.2.1. In vivo function

Cellobiose dehydrogenase was first reported in 1974 and since then scientists have been discussing its *in vivo* function (Kracher, 2016). Among different hypothesis, the one describing a Fenton type reaction in which CDH degrades lignocellulosic biomass by introducing highly reactive hydroxyl radicals was the most accepted for a long time. To be more specific, electrons obtained from oxidation of carbohydrates reduce one electron acceptors such as Fe^{3+}. These reduced species generates hydroxyl radicals in the next reaction

with hydrogen peroxide (Henriksson et al., 2000b; Zamocky et al., 2006; Ludwig et al., 2010; Harreither et al., 2011; Langston et al., 2011).

Today, most of recent publications support the idea that CDHs provide electrons for copper-dependent lytic polysaccharide monooxygenases. The rate of this interenzyme electron transfer goes far beyond the rate of the mentioned Fenton reaction (Hemswort et al, 2015; Kracher et al., 2016). For example, in the catalytic step, oxidation of cellobiose to cellobiono-1,5-lactone leads to reduction of FAD to $FADH_2$. During re-oxidation to FAD electrons are transferred by two internal transfer steps via CDH's cytochrome domain to LPMO. These electrons reduce $Cu^{2+}$to Cu^{1+} and in that way initiate LPMO action. Reduced LPMO catalyzes the oxidative degradation of all major polysaccharides (Morgenstern et al., 2014; Hemswort et al, 2015; Kracher et al., 2016).

2.3.2.2. In vitro electron transfer

CDH belongs to a limited number of oxidoreductases which are, even in their wild-type form, capable of direct electron transfer between the enzyme and an electrode. According to the bioelectrochemical properties of the oxidoreductases, they are characterized as, so called, extrinsic or intrinsic redox enzymes (Ludwig et al., 2013). For the intrinsic ones, several different conditions for direct electron transfer must be fulfilled. In general, the distance between the site of catalytic reaction and the electrode surface must be short enough to enable efficient electron pathway. In the electron transfer pathway from extrinsic enzymes to electrodes, another enzyme is often included. This enzyme acts as a mediator between the active site and a surface area exposed to electrode. If we have in mind CDH's structure, the fact that CDH acts as extrinsic enzyme is clear. From DH domain, the catalytic site, electrons are shifted via built-in mediator, CYT domain, to an electrode (Ludwig et al., 2013).

After the reaction on DH, electrons do not have to be forwarded to natural acceptors. In fact, reduced $FADH_2$ can be reoxidized to FAD by different two or one electron acceptors, meaning that this process does not have to be cytochrome-mediated (Tasca et al., 2011). Electrons can be donated directly to the electrode, but this is doable only for a few CDHs at acidic pH values because FAD is buried too deep in the DH domain. Another possibility for this process is mediated electron transfer using two electron acceptors, quinones or osmium containing polymers. By the laws of electrochemistry, these mediators have to have higher redox potential but they also need to have appropriate size so they can access the well shielded cofactor FAD (Schulz, 2015).

Furthermore, CDHs were formerly described as oxidases, since DH can reduce oxygen to hydrogen peroxide. A number of scientists reported that rate of this reaction was very low. Improvements were observed with higher pH values, comparing to one electron acceptor, CYT, but not with two electron acceptors such as the one stated above. These facts, especially the ability of CDHs to undergo DET via internal electron transfer to CYT are used in different applications of CDHs which will be further discussed in following the subchapter. To be more precise, all CDHs' properties are considered in various possible applications (Schulz, 2015).

2.3.3. Applications

2.3.3.1. Biosensors

Analytes from wide substrate spectrum or wide electron acceptors spectrum are usually subjects of CDHs' application (Table 1) (Ludwig et al., 2010, Ludwig et al., 2013). With higher concentration of substrate more electrons are obtained which results with a higher current increasement. Opposite to that, detection of electron acceptors results with the inversely proportional ratio between the catalytic current and the concentration of analyte (Schulz, 2015).

To this day, the most important CDH biosensors are designed for detection of disaccharides cellobiose and lactose as well as monosaccharide glucose. A CDH biosensor for the detection of cellobiose enables the measurement of the cellobiohydrolase activity on insoluble cellulose, since the reaction product, cellobiose is subjected to CDH oxidation. However, cellobiose causes substrate inhibition because of which lactose is used as standard in these measurements (Ludwig et al., 2013).

Furthermore, very sensitive lactose sensors have found a place in the dairy industry. Depending on many factors such as CDH's origin, sensors have displayed different detection ranges. Among different approaches for the development, a combination of thermometric and amperometric methods resulted in very reliable sensors. Also a DET working mode sensor was recently developed. This kind of sensor is used in measuring time-consuming release of lactose from different drugs. Tracking the release of a widely used filling material in drugs, lactose, leads to better understanding of drug's active component releasement (Knöös et al., 2014).

A third generation glucose biosensor is based on class II CDHs' ability to oxidize glucose. Although the catalytic efficiency of glucose oxidation is not as high as the one for lactose or cellobiose, it is high enough for the construction of biosensors. Unfortunately, the number of people suffering from diabetes is increasing. One of the most important parts of

efficient disease treatment is the reliable measurement of blood sugar levels. Previously developed biosensors are usually based on enzymes like glucose oxidases or glucose dehydrogenases and a redox mediator. Direct electron transfer between intrinsic enzyme as glucose oxidase and an electrode is strongly disfavored which is not case for CDH. Moreover, this allows the development of oxygen-nondependent or redox mediators-nondependent glucose biosensor. As the most promising candidate for such biosensor, is the main topic of this thesis, CDH from *Corynascus thermophilus*. The *Ct*CDH sensor developed in 2011, detects glucose in a linear range between 0.1-30 mM under physiological conditions (Tasca et al., 2011; Zafar et al., 2012; Felice et al., 2013; Ludwig et al, 2013)

An example of the detection of electron acceptors is the CDH based biosensor for quinones. During detection of these potential electron mediators and their reduced counterparts, a higher potential must be applied. Also these biosensors are not as selective as the ones for carbohydrates (Ludwig et al., 2013)

Table 1. CDH biosensors (adapted from Ludwig et al., 2013)

Analyte	**Detection limit**	**Mediator/ enhancer**	**Electrode modification**	**Electrode material**	**CDH**
Cellobiose	0.5 μM	No	adsorption	spectroscopic graphite	*Mt*CDH
Lactose	1 μM	No	adsorption	spectroscopic graphite	*Mt*CDH
Glucose	0.01 mM	No	cross-linked+ SWCNTs	screen-printed carbon electrode	*Ct*CDH
Glucose	0.05 mM	No	cross-linked+ SWCNTs	spectroscopic graphite	*Ct*CDH
Hydroquinone	0.75 nM	cellobiose	adsorption	spectroscopic graphite	*Pc*CDH
Catechol	1 nM	cellobiose	adsorption	spectroscopic graphite	*Pc*CDH

2.3.3.2. Two phase reaction

Another interesting possible application of CDH is in organic chemistry. Its ability to generate H_2O_2 *in-situ* can be used in various processes like cotton bleaching, in detergents or

in pulp and paper industry (Pricelius et al., 2009; Sygmund et al., 2013). As it has already been stated, the main drawback of CDH is its low affinity for oxygen as electron acceptor. Therefore a CDH variant with an increased affinity for oxygen has to be used for H_2O_2 production. The proposed applications are eco-friendly comparing to the standard (chemical-based) ones since there are no toxic by-products or excessive consumption of water and energy (Sygmund et al., 2013). Here we propose a new possible application of CDH based on *in situ* production of H_2O_2 . CDH is used together with enzyme, unspecific peroxygenase (UPO) in a cascade reaction. This process is based on the fact that peroxidases *in vivo* depend on the activity of auxiliary enzymes which can provide hydrogen peroxide required for their activation (Martínez et al., 2014).

Agrocybe aegerita unspecific peroxygenase belongs to a new type of peroxide-using, heme-thiolate enzymes that acts as a self-sufficient monooxygenases in the presence of enough hydrogen peroxide (Molina-Espeja et al., 2014). It catalyzes stereoselective benzylic hydroxylation of alkylbenzenes as well as C1-C2 epoxidation of styrene derivatives. For some benzylic hydroxylation products ((*R)-1*-phenylalkanols), enantiomeric excess is reported to be higher than 99%. Due to few reasons, among which its stability and efficiency, several publications point out that no other catalyst is as efficient as UPO in these reactions (Kluge et al., 2012), while others believe that the common problem of all heam peroxidases is the enzyme inactivation by excess peroxide and this should be specially considered (Hofrichter and Ullrich, 2013).

Optically pure α-hydroxy alkylbenzenes and epoxides are widely used as building blocks in industry of fine chemicals, pharmaceuticals, antibiotics and odorous substances. Since classical chemical productions of these compounds are conducted in extreme and harsh reaction conditions, often economically and environmentally problematic, there are different efforts to avoid those kinds of disadvantages (Kluge et al., 2012).

3. MATERIALS AND METHODS

3.1. MATERIALS AND METHODS FOR THE TRANSFORMATION PROCESS

3.1.1. Strains

The fungal strain *Aspergillus niger* D15#26 (pyrG-) had been used for the heterologous expression of the *Ct*CDH and *Ao*CDH III genes. pAN52.4 vector carrying the gene for the cellobiose dehydrogenase from *Corynascus thermophilus* C310Y and pan56.1 with *Aspergillus oryzae* CDH III were produced in bacterial strain *Escherichia coli* NEB5α as well as the pAB4.1 plasmid with the *pyrG* gene from *Aspergillus niger*.

3.1.2. Chemicals

The list of chemicals used in the first part of this thesis is shown in Table 2.

Table 2. List of chemicals

Chemical	**Manufacturer**
Acetic acid	Fluka, Switzerland
Agar-Agar, Kolbe I	Roth, Germany
Ammonium sulfate	Roth, Germany
Ampicillin sodium salt	Roth, Germany
Aurin tricarboxylic acid ammonium salt, ATA	Merck, Germany
Bradford reagent	Sigma, USA
Boric acid, ≥ 99%	Sigma, USA
Calcium chloride dihydrate	Sigma, USA
D(+)-cellobiose	Fluka, Switzerland
Citiric acid	Sigma, USA
Cobalt (II) chloride hexahydrate	Sigma, USA
Copper (II) sulphate	Sigma, USA
Cytocrome c, from equine heart	Sigma, USA
Disodium hydrogen phosphate dihydrate	Fluka, Switzerland
Ethanol (96 %)	Sigma, USA
EDTA Ethylenediaminetetraacetic acid disodium salt dihydrate	Sigma, USA
α-D(+)-Glucose monohydrate	Roth, Germany
Glycine	Sigma, USA
Iron (II) sulfate heptahydrate	Sigma, USA
D(+)-Lactose monohydrate	Roth, Germany
Magnesium sulfate heptahydrate	Sigma, USA
Manganese (II) chloride tetrahydrate	Roth, Germany
Polyethylene glycol 6'000	Fluka, Switzerland

Potassium chloride	Fluka, Switzerland
Potassium dihydrogen phosphate	Roth, Germany
Sodium chloride	Roth, Germany
Sodium hydroxide	Fluka, Switzerland
Sodium molbydate dihydrate	Fluka, Switzerland
Sodium nitrate	Roth, Germany
D-Sorbitol	Sigma, USA
Tween 20	Sigma, USA
Zinc sulfate heptahydrate	Sigma, USA
Peptone from casein	Fluka, Switzerland
Yeast extract	Fluka, Switzerland
2-ethanesulfonic acid	Sigma, USA
2,6-dichloroindophenol	Fluka, Switzerland

3.1.3. Culture media

LB (Luria-Bertani) Lysogeny broth with ampicilin

The ingredients of 100 mL Luria-Bertani medium: 1 g NaCl, 1 g peptone from casein, 0.5 g yeast extract and 100 mL of distilled water. Everything was mixed and autoclaved at 121°C for 20 min. 100 μL of an ampicillin solution (100 mg mL^{-1}) was added to the medium after the medium had been cooled down.

Minimal medium plates

In order to get 500 mL of the minimal medium agar we autoclaved (20 min/121°C) 480 mL of distilled water with 8 g of agar. After that, the medium was placed in 60°C water bath for 10 minutes. Previously sterilized solutions, 10 mL of Asp A+N, 10 mL of 50% glucose, 1 mL of 1 M $MgSO_4$ and 100 μL of trace elements solution were then added under sterile conditions.

Minimal medium + sorbitol plates

The composition of 500 mL minimal medium + sorbitol plates was the same as the one stated above, except before the sterilization, 200 mL of 3 M sorbitol had been added.

Minimal medium for screening after transformation

The composition of 1000 mL of the medium was as follows: 28.4 g Na_2HPO_4, 20 mL of Asp A-P, 2 mL of 1 M $MgSO_4$ were added to 880 mL of distilled water. Before the sterilization (20 min/121°C), the pH had been adjusted to 5.5 with 1 M citric acid. After the

autoclaving, 100 mL of 50% glucose and 1 mL of the trace elements solution were added to the medium under sterile conditions.

Complete medium plates

For the volume of 500 mL we added 1 g of peptone from casein, 2.5 g of yeast extract and 8 g of agar to 480 mL of distilled water. The mixture was autoclaved for 20 min at 121 °C after which it was cooled down to 60 °C. Just before plates were poured, 10 mL of Asp A+ N, 10 mL of 50% glucose, 1 mL of 1 M $MgSO_4$ and 0.5 mL of the trace elements solution, had been added to the medium under sterile conditions.

Complete medium (liquid)

The composition of 500 mL complete medium was the same as the composition of complete medium plates. The only difference is that the liquid medium did not contain any agar.

3.1.4. Buffers and solutions

3.1.4.1. The composition of the solutions used for the transformation, screening and production

AspA + N solution [50x]

For 500 mL:

$NaNO_3$	148.8 g
KCl	13.05 g
KH_2PO_4	37.4 g

Salts were dissolved in distilled water. The pH value was adjusted to 5.5 with NaOH. The solution was autoclaved for 20 min at 121°C.

AspA -P solution [50x]

For 500 mL

$NaNO_3$	148.8 g
KCl	13.05 g

Salts were dissolved in distilled water. The pH value was adjusted to 5.5 with NaOH. The solution was autoclaved for 20 min at 121°C.

1 M $CaCl_2$

14.7 g $CaCl_2$•2 H_2O per 100 mL distilled water.

The solution was autoclaved for 20 min at 121°C.

$CaCl_2$ 1700 solution

For 500 mL

$CaCl_2$•2 H_2O	19.8 g
NaCl	17.5 g

The volume was filled up to 500 with distilled water. The solution was autoclaved for 20 min at 121°C.

1 M Citric acid

96.05 g of citric acid per 500 mL distilled water.

The solution was autoclaved for 20 min at 121°C.

50% glucose solution

50 g glucose per 100 mL distilled water.

The solution was autoclaved for 30 min at 110°C.

1 M MgSO4

24.6 g $MgSO_4$•7H_2O per 100 mL distilled water.

The solution was autoclaved for 20 min at 121°C

PEG 1700

For the volume of 20 mL:

PEG 6000	5 g
1M CaCl2	1 mL

1M Tris, pH 7.5	200 μL

The solution was sterilized by filtration through a sterile 0.2 μm filter.

3 M Sorbitol

163.95 g of *D*-Sorbitol was gradually dissolved in 150 mL of distilled water, after which the volume was filled up with distilled water to 300 mL. The solution was autoclaved for 20 min at 121°C.

STC 1700 solution

For the volume of 500 mL:

3M Sorbitol	200 mL
1M Tris pH 7.5	5 mL
$CaCl_2$•2 H2O	3.7 g
NaCl	1 g

The volume was filled up to 500 mL with distilled water and autoclaved for 20 min at 121°C.

Trace elements solution

$ZnSO_4$•7 H_2O	2.2 g
H_3BO_3	1.1 g
$MnCl_2$•4 H_2O	0.5 g
$FeSO_4$•7 H_2O	0.5 g
$CoCl_2$•6 H_2O	0.17 g
$CuSO_4$•5 H_2O	0.16 g
$NaMoO_4$•2 H_2O	0.15 g
Na_2EDTA	6.48 g

EDTA was dissolved before other salts had been added in 80 mL distilled water. The pH value was adjusted to 8.0 with KOH. The volume was filled up to total 100 mL with distilled water. The solution was sterilized by filtration through a sterile 0.2 μm filter.

1 M Tris, pH 7.5

To 60.6 g Tris 490 mL of distilled water was added. The pH value was adjusted to 7.5 with HCl. The volume was filled up to 500 mL with distilled water and autoclaved for 20 min at 121°C.

3.1.4.2. Buffers and solutions used during the purification

Buffer A

50 mM sodium acetate buffer pH 5.5 containing ammonium sulfate

Acetic acid	4.29 mL
Saturated ammonium sulfate solution	300 mL

The acid was added to 375 mL deionized water, after ammonium sulfate solution was added to get 20 % saturated solution. The pH value was adjusted to 5.5 using 4M NaOH. The volume was filled up to 1500 mL with distilled water.

Buffer B

Acetic acid	4.29 mL

The acid was added to 375 mL deionized water. The volume was filled up with distilled water to around 1400 mL. The pH value was adjusted to 5.5 using 4M NaOH. The final volume of solution was adjusted to 1500 mL with deionized water.

0.5 M NaCl

NaCl	43.83 g

Salt was dissolved in distilled water; the final volume was 1.5 L.

3.1.4.3. Solutions used in activity assays and the characterization steps

Bradford reagent

10 mL of Bio-Rad protein assay dye reagent concentrate was diluted in 40 mL of high quality distilled water, after which it was filtered through a paper filter.

Cytochrome *c* solution

Considering our needs, we dissolved 3.1, 6.2 or 12.4 mg of cytochrome *c* in 0.25, 0.5 or 1 mL of distilled water. The solution was stored at 4°C in the dark for three days.

0.5 mM cellobiose solution

Cellobiose	4.278 mg
a) McIlvaine buffer pH 5.5	25 mL
b) McIlvaine buffer pH 7.5	25 mL

20 mM cellobiose solution

Cellobiose	0.1712 g
a) McIlvaine buffer pH 5.5	25 mL
b) McIlvaine buffer pH 7.5	25 mL

DCIP solution

87.03 mg of 2,6-dichloroindiphenol (DCIP) was dissolved in 10 mL 96% ethanol by stirring (30 min, 50°C) in a volumetric flask. After complete dissolution, the final volume was adjusted to 100 mL with distilled water. The solution was kept in the dark at 4°C.

300 mM lactose solution

Lactose	10.81 g
Distilled water	100 mL

Lactose was dissolved at 50°C. The solution was stored for max 1 month at 4°C.

McIlvaine buffer

A solution: Citric acid 19.21 g per 1L

B solution: Na_2HPO_4 35.6 g per 1L

In order to get 100 mL of the buffer with desired pH value, we added corresponding amount of the solution B as shown in Table 3 and filled up to the final volume with the buffer A.

Table 3. Volume of the solution B to which corresponding amount of the solution A needs to be added to get a specific pH value

pH	**.0**	**.1**	**.2**	**.3**	**.4**	**.5**	**.6**	**.7**	**.8**	**.9**
2	-	-	-	-	-	-	10.8	13.2	15.6	18.1
3	20.6	22.6	24.7	26.6	28.5	30.3	32.2	33.9	35.5	37.1
4	38.6	40.0	41.4	42.7	44.0	45.4	46.7	48.0	49.3	50.4
5	51.5	52.6	53.6	54.7	55.8	56.9	58.0	59.2	60.5	61.8
6	63.2	64.6	66.1	67.7	69.3	71.0	72.8	74.8	77.2	79.8
7	82.4	85.6	85.6	88.8	90.7	92.1	93.6	94.6	95.7	-

3.1.5. Equipment

Besides the laboratory dishes, the equipment described below was used:

- Analytical balance (AW-4202, Sartorius group, Germany)
- Autoclave (VarioClav Classic, Thermo Fisher Scientific, USA)
- Centrifuge (Avanti J-26 XP, Beckman Coulter Inc., USA)
- Centrifuge (Centrifuge 5810 R, Eppendorf, Germany)
- Chromatography system (ÄKTA explorer 10, GE Healthcare Life Sciences, USA)
- Digital dry bath (Accublock, Labnet International, USA)
- Incubation shaker (Unitron, Infors HT, Schwitzerland)
- Incubator (BE200 Bench top incubator, Memmert, Germany)
- Laminar flow cabinet (Safe 2020, Thermo Fisher Scientific, USA)
- Magnetic stirrer (RCT basic, IKA, Germany)
- pH meter (744, Metrohm, Switzerland)
- Spectrophotometer (DU 800, Beckman Coulter Inc., USA)
- Table centrifuge (Centrifuge 5415 R, Eppendorf, Germany)
- Table shaker (Titramax 100, Heidolph Instruments, Germany)
- Technical balance (AW-224, Sartorious group, Germany)
- Ultrasonic bath (Sonorex super, Bandelin, Germany)
- UV/Vis spectrometer (Lambda 35, Perkin Elmer, USA)
- Vortex (Vortex genie 2, Scientific Industries, USA)
- Water bath (TW 12, Julabo, Germany)

3.2. METHODS: TRANSFORMATION PROCESS

3.2.1. Cultivation of *Escherichia coli* NEB5α with pAN52.4-*AoCDH III*, pAN52.4-*CtCDH* and pAB4.1

Escherichia coli NEB5α with pan56.1-*AoCDH III*, *Escherichia coli* NEB5α with pan52.4-*CtCDH* and *Escherichia coli* pAB4.1 had previously been cultivated on lysogeny broth agar plates with ampicillin. Set of eprouvettes with 2 ml lysogeny broth + ampicilin was than inoculated with the pure culture of the strains described above and cultivated on a shaker at 140 rpm and 37°C for 12 h.

After the cultivation in eprouvettes, 50 mL of the lysogeny broth with ampicillin was inoculated with the content from epprouvettes and cultivated in the same cultivation conditions as for the eprouvettes.

3.2.2. Isolation of plasmids

The isolation of the plasmids from NEB5α with pAN56.1-*Ao*CDH III, pAB 4.1 and pAN52.4-*Ct*CDH was carried out using plasmid isolation kit: QIAGEN Plasmid Plus Midi Kit (Appendix). Bacterial cells were harvested by centrifuging, after which they were resuspended in Buffer P1. Buffer P2 was added to the suspension and mixed gently. The mixture was left at room temperature, after which Buffer S3 was added. The mixture was then transferred to the QIAfilter Cartridge and incubated again at room temperature. Filtration of the cell lysate was carried out and Buffer BB was added. The mixture was transferred to a QIAGEN Plasmid Plus spin column on the QIAvac 24 Plus after which it was vacuum filtered. The DNA washing was carried out using Buffer ETR and Buffer PE. Vacuum filtration was then repeated. In order to eliminate residual buffer, the column was centrifuged. The QIAGEN Plasmid Plus spin column was transferred into a clean 1.5 mL tube. For the DNA elution 150 µL of Buffer EB was added to the spin column, the mixture was incubated at room temperature and then centrifuged.

3.2.3. Cultivation of expression host, *Aspergillus niger* D15#26 (pyrG-)

Aspergillus niger D15 spores from complete medium agar plates were collected with Tween 20 solution. Two 1 L Erlenmeyer flasks with 250 mL complete medium were inoculated with the spore solution to get the final concentration of $2*10^6$ spores mL^{-1}. The cultivation lasted for 12 h on a shaker at 150 rpm and 30°C.

3.2.4. *Aspergillus niger* transformation

The process was carried out as protoplast-mediated transformation (Arentshorst et al., 2012). Right before the transformation, the PEG solution was prepared and 300 mg of lyzing enzyme Caylase C4 was dissolved in 50 mL $CaCl_2$ 1700 buffer. Both solutions were then sterilized by filtration through a sterile filter.

In order to get protoplasts, the *Aspergillus niger* mycelium, previously cultivated in complete medium flasks, was filtered through a sterile miracloth filter. It was then washed twice with about 100 mL of $CaCl_2$ 1700. The mycelium was weighed and 3 g of the wet mycelium was re-suspended in lyzing enzyme solution and left on shaker at 80 rpm and 30°C for 2 h.

Since protoplasts are very fragile, they had been handled carefully. After 2 hours, protoplasts were filtered through the sterile miracloth into a 50 mL tube, diluted to ½ with cold STC 1700 buffer and left on ice for 10 min. We centrifuged them at 2500 rpm for 15 min at 4°C and discarded the supernatant; afterward a 1 mm film was visible at the bottom. The film was re-suspended in 25 mL of cold STC 1700 buffer and centrifuged at 3000 rpm for 10 min at 4°C. Two washes with STC 1700 buffer were made, after which protoplasts were re-suspended the in 1 mL STC 1700 buffer.

The transformation was carried as follows: in a 50 mL tube, 1 µL of DNase inhibitor ATA, 2 µL of pAB4.1, 10 µL of desired gene solution and 150 µL of freshly prepared protoplasts were added. The content was gently mixed and left at room temperature for 30 min. To avoid damaging protoplasts, six drops of the PEG solution were added on the wall of the tube, mixed and then 850 µL PEG was added. Since the PEG solution is toxic to protoplasts, incubation time with the stated solution was exactly 5 min. Afterwards, the mixture was diluted in 10 mL of STC 1700 and centrifuged at 3000 rpm for 10 min. The supernatant was drained whereas the residue was re-suspended in of STC 1700, generating the total volume of 500 µL. Minimal medium plates with sorbitol, previously warmed up at 30°C, were inoculated with the transformation mixture. Sealed plates with parafilm were left in the oven at 30°C, until growth was visible.

3.2.5. *Aspergillus niger Ct*CDH screening

Few days after the transformation, before they sporulated, transformed single colonies had been transferred to small minimal medium plates without sorbitol. After plates were completely covered with the sporulating mycelium, normal minimal medium plates were

inoculated with the spore solution from those plates. With this subcultivation on normal plates we wanted to ensure enough spores for the liquid medium inoculation.

The screening process is performed in order to see which transfomants will have the best expression of the desired enzyme. The screening was conducted in three 300 mL non-baffled flasks. Spores from minimal medium plates were collected using a Tween 20 solution. Each flask with 100 mL of the minimal medium for screening was inoculated with the spore solution. The cultivation in screening medium lasted 10 days on a shaker with a shaking speed of 160 rpm and at 30°C. From the third day the pH value of the broth was adjusted to 5.5 with citric acid. From the sixth to the tenth day, samples were taken and tested for the DCIP activity.

After the screening process, a sample was given for a sequence analysis in order to confirm that produced enzyme is *Ct*CDH. For that purpose, an SDS-PAGE electrophoresis was run with the crude extract sample after which band was cutted and sent for the analysis (Appendix 1).

3.2.6. *Aspergillus niger Ct*CDH cultivation

After the screening process, one, the most promising producer of *Ct*CDH was chosen for a production. Five minimal medium agar plates were inoculated with the spore solution of this colony, collected before the screening. With this step we got enough inoculum for production which we used to inoculate fifteen non-baffled flasks with 300 mL of minimal medium for screening. The final concentration of spores in medium was $2*10^6$ spores mL^{-1}. The cultivation was carried out for 10 days on shakers at shaking speed of 160 rpm and at 30°C. From the third day the pH value of the medium was set to 5.5 with 1 M citric acid. From the sixth to the tenth day, samples were taken and tested for DCIP activity.

3.2.7. Enzyme purification

The purification of the fermentation broth was carried using the ÄKTA explorer 10, in two step procedure: hydrophobic interaction chromatography and ion exchange chromatography.

On the day when the activity reached its maximum, harvest was performed by filtration through a miracloth. Saturated ammonium sulfate solution was slowly added to the clarified broth to get a 20% saturated solution.

In the first step, solution was applied on a PHE Sepharose 6 fast flow column (70 mL resin and equipment are from GE Healthcare) equilibrated with 50 mM sodium acetate buffer pH 5.5 containing 20 % (saturation) ammonium sulfate. The elution of proteins was done within a linear gradient from 20 to 0% ammonium sulfate in 3 column volumes, and collected in 11 mL fractions. We pooled the active fractions after which we diafiltrated the solution using a hollow fiber cross-flow module (Microza UF module SLP-1053, 10 kDa cut-off, Pall Corporation).

In the second step, the partially deionized enzyme solution (4 mS cm^{-1}) was applied to a 20 mL Q15-Source column, previously equilibrated with 50 mM sodium acetate buffer. Proteins were eluted within a linear salt gradient from 0 to 0.5 M NaCl in 15 CV. The active fractions were concentrated and the buffer exchanged using a polysulfonate spin column.

During the purification the activity was measured with the DCIP and the cytochrome *c* assays.

3.2.8. Analytical methods

3.2.8.1. Bradford protein assay

This colorimetric assay is based on an absorbance maximum shift of an acidic solution of the Coomassie Brilliant Blue G-250 dye from 465 nm to 595 nm when it binds to proteins (Bradford, 1976). All measurements were performed with the Beckmann DU-800 spectrophotometer. The measurement was performed in a way that to 15 μL of the protein sample 600 μL of the Bradford reagent was added. The mixture was left in the dark, at room temperature, for 12 min and then the absorbance of the samples was measured at 595 nm. The software calculates protein concentrations from previously prepared standard curve.

3.2.8.2. Cellobiose dehydrogenase activity assay using DCIP

This method is a standard method for CDH activity measurements in crude extracts or partially purified samples. In order to avoid substrate inhibition caused with its natural substrate cellulose, lactose or cellobiose is used instead. The activity is detected by the reduction of DCIP (the electron acceptor) which decolorizes the initially blue assay mixture (Bao et al., 1993, Baminger et al., 2001). All measurements were performed with the Lambda 35 UV/Vis spectrometer and its accompanying software.

Pipetting protocol was as follows: 100 µL of the DCIP solution, 100 µL of the substrate solution (300mM lactose or cellobiose) and 780 µL of the buffer (McIlvaine pH=5.5). Cuvettes with mixtures were then temperated for minimum 20 min at 30°C in a water bath. The reaction began when 20 µL of the sample was added to the cuvette, which was already placed in the sample holder. The absorbance was recorded at 520 nm for 180 sec. The software calculates activity from the negative slope (decrease) of the absorption.

3.2.8.3. Cellobiose dehydrogenase activity assay using cytochrome c

This method is a reference method for CDH determination, but only in crude extracts or partially purified samples. Opposite to the DCIP method, only the activity of the holoenzyme CDH is determined. The substrates which oxidize in the reaction are lactose or cellobiose. The electron acceptor, cyt *c* is reduced during the reaction which causes the color change from orange to a more pinkish tone (Baminger et al., 2001). Measurements were performed with the same spectrophotometer as for the DCIP activity assay.

The measurement was carried in a way that 20 µL of the cyt *c* solution, 100 µL of the substrate solution and 860 µL of the buffer (McIlvaine, pH= 7.5) were pipetted, respectively, in a cuvette. The mixture was incubated at 30°C in a water bath, minimum 20 min and then transferred to the sample holder. The reaction started when 20 µL of the sample was added to the solution. The absorbance was measured at 550 nm for 180 sec. The software calculates activity from the positive slope (increase) of the absorption.

3.2.8.4. Kinetics constants assay

In order to determine kinetic constants of *Ct*CDH for cellobiose we measured *Ct*CDH activity with different concentrations of cellobiose. Measurements were performed using both, the DCIP and the cyt *c* assays. For the DCIP activity we used McIlvaine buffer pH 5.5 and for the cyt *c* assay McIlvaine buffer pH 7.5. Samples were prepared as stated in Table 4. for DCIP and in Table 5 for cyt *c*.

Table 4. Sample preparation for the DCIP assay

Cellobiose [µM]	**Substrate [µL]**	**Stock solution [mM]**	**DCIP [µL]**	**Enzyme [µL]**	**Buffer [µL]**
10	20	0.5	100	20	860
25	50	0.5	100	20	830
50	100	0.5	100	20	780
100	200	0.5	100	20	680

200	400	0.5	100	20	480
600	30	20	100	20	850
2000	100	20	100	20	780
5000	250	20	100	20	630
10000	500	20	100	20	380

Table 5. Sample preparation for the cyt *c* assay

Cellobiose [μM]	**Substrate [**μL**]**	**Stock solution [**mM**]**	**Cyt *c* [**μL**]**	**Enzyme [**μL**]**	**Buffer [**μL**]**
5	10	0.5	100	20	950
25	50	0.5	100	20	910
50	100	0.5	100	20	860
150	300	0.5	100	20	660
250	500	0.5	100	20	460
500	25	20	100	20	935
800	40	20	100	20	920
1200	60	20	100	20	900
2500	125	20	100	20	835
5000	250	20	100	20	710

3.3. MATERIALS AND METHODS FOR TWO-PHASE BIOCONVERSION

3.3.1. Enzymes

Unspecific peroxygenase (UPO) from the edible mushroom *Agrocybe aegerita* was expressed in *Pichia pastoris* and purified previously. For this work different variants of enzyme cellobiose dehydrogenase (CDH) from *Myriococcum thermophilum* were used. The dehydrogenase domain of CDH was previously engineered to achieve higher oxygen affinity. *Pichia pastoris* was used as an expression host. N700S CDH variant has a single mutation at 700th residue, where asparagine is substituted with serine. In CDH variant N748G asparagine at 748th residue is replaced with glutamine and in T750Q variant at 750th residue threonine is substituted with glutamine. CDH variants N748G/T750Q and N700S/N748G have mutations which are the combination of the mutations described above. CDH N700S/N748G/T750Q variant has three mutations, already stated and described above.

3.3.2. Chemicals

The list of chemicals used in the second part of this thesis is shown in Table 6.

Table 6. List of chemicals

Chemical	Manufacturer
ABTS, diammonium salt	Amresco, USA
Acetic acid	Fluka, Switzerland
Acetonitrile	Merck, USA
Bradford reagent	Sigma, USA
D(+)-cellobiose	Fluka, Switzerland
Citric acid	Sigma, USA
Disodium hydrogen phosphate dihydrate	Fluka, Switzerland
Ethanol (96 %)	Sigma, USA
Ethylbenzene	Aldrich, USA
Hydrogen peroxide 30%	Fluka, Switzerland
D(+) Lactose monohydrate	Roth, Germany
Methanol	Roth, Germany
Potassium dihydrogen phosphate	Roth, Germany
Potassium hydroxide	Fluka, Switzerland
Sodium carbonate anhydrous	Sigma, USA
1-Phenylethanol	Aldrich, USA
2,6-dichloroindiphenol	Fluka, Switzerland

3.3.3. Composition of buffers and solutions

McIlvaine buffer

A solution: Citric acid 19.21 g per 1L

B solution: Na_2HPO_4 35.6 g per 1L

The buffer was prepared as previously described in 3.4.7

100 mM Potassium phosphate buffer (KPP) pH 5.0

Potassium dihydrogen phosphate 6.8045 g per 500 mL

The salt was dissolved in 300 mL of distilled water, the pH value was adjusted to 5.0 with KOH, afterward the volume was filled up to 500 mL with distilled water.

100 mM Potassium phosphate buffer (KPP) pH 7.0

Potassium dihydrogen phosphate 6.8045 g per 500 mL

The salt was dissolved in 300 mL of distilled water, pH was adjusted to 7.0 with KOH, afterward the volume was filled up to 500 mL with distilled water.

200 mM sodium phosphate/citrate buffer pH 4.4

For 200 mL:

7.12 g of $Na_2HPO_4x2H_2O$ was dissolved in 150 mL of distilled water and then titrated with 10 % citric acid to pH 4.4. The volume was filled up to 200 mL with distilled water.

ABTS reagent

10 mM	10.3 mg of ABTS was dissolved in 2 mL distilled water
3 mM	3.09 mg of ABTS was dissolved in 2 mL distilled water

The reagent was always freshly prepared. It is stable for 12 hours at 4 °C.

Bradford reagent

The reagent was prepared as previously described in 3.1.4.3.

HRP reagent

10 mg of horseradish peroxidase was dissolved in 1 mL distilled water which equals 2000 U mL^{-1}. In order to get 1 U mL^{-1}, the reagent was diluted 1:2000.

Cytochrome *c* solution

The preparation of the solution has already been described in 3.1.4.3.

DCIP solution

The solution was prepared as previously described in 3.1.4.3.

Hydrogen peroxide solution

According to our needs 30 % H_2O_2 solution was diluted 1:1000 or 1:10000.

300 mM ethylbenzene solution

Ethylbenzene	443.2 µL
Methanol	14.44 mL

300 mM lactose solution

The solution was prepared as it has already been described in 3.5.

Cellobiose solution

10 mM	Cellobiose	0.1712 g
100 mM	Cellobiose	1.712 g

In order to get cellobiose solution with desired concentration we dissolved corresponding amount in McIlvaine buffer pH 7. The final volume was 50 mL.

3.3.4. Equipment

During this work, besides the laboratory dishes, the following equipment was used:

- Analytical balance (AW-4202, Sartorius group, Germany)
- Bioreactor (built-in-house)
- Digital dry bath (Accublock, Labnet International, USA)
- Flow console (Applikon, Holland)
- Fiber-Optic Oxygen Microsensor (PreSens, Germany)
- HPLC (Summit, Thermo Fisher Scientific, USA)
    - Reverse phase column (Discovery C18, Supelco, USA)
    - UV/Vis detector (Dionex Ultimate 3000, Thermo Fisher Scientific, USA)
- Magnetic stirrer (RCT basic, IKA, Germany)
- Oxygen meter (Microx TX3, PreSens, Germany)
- pH meter (744, Metrohm, Switzerland)
- pH meter (BioController, Applikon, Holland)
- Pump (7549-20, Cole-Parmer, UK)
- Spectrophotometer (DU 800, Beckman Coulter Inc., USA)
- Table centrifuge (Centrifuge 5415 R, Eppendorf, Germany)
- Thermo-shaker (, Eppendorf, Germany)
- Technical balance (AW-224, Sartorious group, Germany)
- Ultrasonic bath (Sonorex super, Bandelin, Germany)
- UV/Vis spectrometer (Lambda 35, Perkin Elmer, USA)
- Vortex (Vortex genie 2, Scientific Industries, USA)

- Water bath (TW 12, Julabo, Germany)
- Water bath (F10, Julabo, Germany)

3.4. METHODS - BIOCONVERSION PROCESS

3.4.1. Analytical methods

3.4.1.1. Bradford protein assay

Measurements were performed as described in 3.2.8.1.

3.4.1.2. H_2O_2 assay

In order to monitor H_2O_2 concentrations generated during the reaction, we used a previously prepared calibration curve for hydrogen peroxide concentrations. For the calibration curve we prepared different concentrations of H_2O_2 as stated in Table 7.

Table 7. Samples preparation for the standard curve

H_2O_2	**Stock solution 1:1000 30 % H_2O_2**	**KPP Buffer pH 7.0**
mM	µL	µL
0.1	10	Filled up to 1 mL
0.25	26	Filled up to 1 mL
0.5	53	Filled up to 1 mL
0.75	77	Filled up to 1 mL
1	103	Filled up to 1 mL
1.5	154	Filled up to 1 mL
2	204	Filled up to 1 mL

In a cuvette we placed 100 µL of ABTS reagent, 20 µL HRP reagent, 860 µL KPP buffer pH=5.5 and 20 µL of corresponding H_2O_2 dilution and then left the mixture at room temperature for twelve minutes. The absorbance of samples was measured at 420 nm with Beckmann DU-800 spectrophotometer after which the calibration curve was plotted.

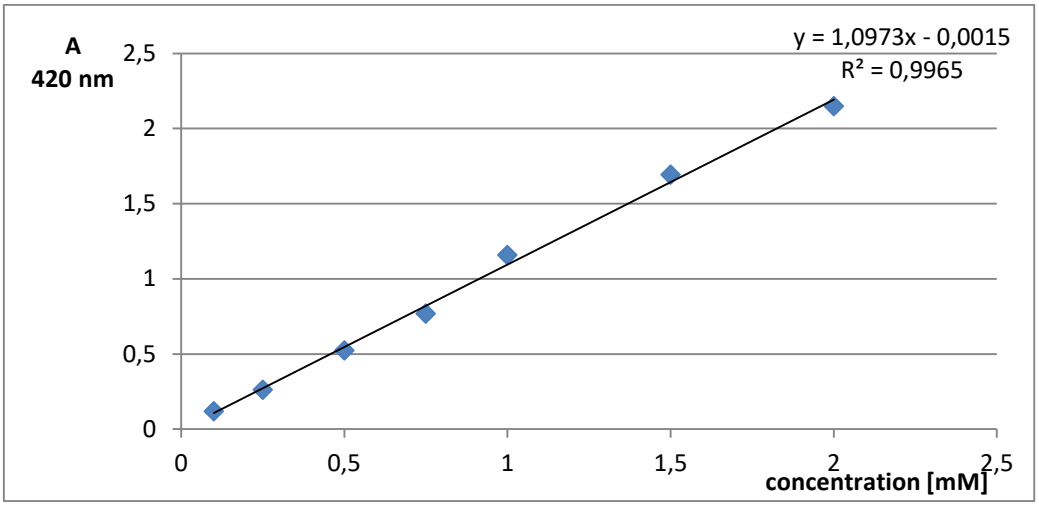

Figure 3. Calibration curve for hydrogen peroxide

3.4.1.3. Peroxidative and peroxygenase activity assay using ABTS-UPO activity

This method is used for peroxygenase activity measurements in crude extracts or partially purified samples. During this oxygen dependent reaction ABTS is oxidized to the ABTS cation radical wherein colorless reaction mixture becomes green (Michal et al, 1983). All measurements were performed with the Lambda 35 UV/Vis spectrometer and its accompanying software.

Pipetting protocol was as follows: 100 µL of the 3 M ABTS solution, 500 µL of the 200 mM sodium phosphate/citrate buffer (pH=4.4), 10 µL of 200 mM H_2O_2 solution and 370 µL of distilled water. Cuvettes with mixtures were then temperated for minimum 20 min at 30°C in a water bath. The reaction began when 20 µL of the sample was added to the cuvette, which was already placed in the sample holder. The absorbance was recorded at 420 nm for 180 sec. The activity was calculated automatically by the software.

3.4.1.4. Cellobiose dehydrogenase activity assay using DCIP

The method is already described in the section 3.2.8.2.

3.4.1.5. Phenylethanol analysis

Phenylethanol was analyzed using HPLC (Summit, Thermo Fisher Scientific, USA). All measurements were performed with the Supelco Discovery C18 reverse phase column in a combination with UV-Detector at 254 nm. Conditions were as follows: 20 % acetonitrile as mobile phase, flow rate 0.2 mL min^{-1}, temperature 30 °C and injection volume 20 µL.

Phenylethanol concentrations of 1 g L^{-1}, 0.5 g L^{-1}, 0,1 g $L^{-1}$and 0.05 g L^{-1}were used for the preparation of the calibration curve. All dilutions were prepared with mobile phase.

3.4.1.6. Kinetic characterization of UPO

In order to obtain more information about substrate inhibition of UPO we analyzed the specific activity of UPO at different hydrogen peroxide concentrations. Samples were prepared as stated in Table 8.

Table 8. Preparation of samples for UPO's kinetics

Final conc. H_2O_2	**Stock solution 1:1000 30 % H_2O_2**	**300 mM ethylbenzene in methanol**	**1:200 UPO**	**KPP Buffer pH 7.0**
mM	µL	µL	µL	µL
0.01	10.2 of 1:10000 30 % H_2O_2	100	20	Filled up to 1 mL
0.05	51.05 of 1:10000 30 % H_2O_2	100	20	Filled up to 1 mL
0.10	10.2 of 1:1000 30 % H_2O_2	100	20	Filled up to 1 mL
0.25	25.5 of 1:1000 30 % H_2O_2	100	20	Filled up to 1 mL
0.50	51.05 of 1:1000 30 % H_2O_2	100	20	Filled up to 1 mL
0.75	76.6 of 1:1000 30 % H_2O_2	100	20	Filled up to 1 mL
1	102.1 of 1:1000 30 % H_2O_2	100	20	Filled up to 1 mL
1.5	154.5 of 1:1000 30 % H_2O_2	100	20	Filled up to 1 mL
2	204.2 of 1:1000 30 % H_2O_2	100	20	Filled up to 1 mL
5	51.05 of 1:100 30 % H_2O_2	100	20	Filled up to 1 mL
10	102.1 of 1:100 30 % H_2O_2	100	20	Filled up to 1 mL
20	204.2 of 1:100 30 % H_2O_2	100	20	Filled up to 1 mL
30	306.3 of 1:100 30 % H_2O_2	100	20	Filled up to 1 mL
40	408.4 of 1:100 30 % H_2O_2	100	20	Filled up to 1 mL
50	510.5 of 1:100 30 % H_2O_2	100	20	Filled up to 1 mL

Each dilution was incubated exactly three minutes at 30 °C in a thermoshaker. In order to stop the reaction tubes were immediately transferred to 99 °C for 3 min or 100 µL of 4M acetic acid was added. Afterwards, the samples were centrifuged for 5 min at full speed. Phenylethanol concentrations were analyzed by HPLC as described above.

3.4.2. Characterization of CDH variants

Specific activity of CDH variants described in 3.3.1.was measured with different oxygen concentrations. Measurements were carried out in a way that 10 mM cellobiose solution in two test tubes was bubbled with oxygen and nitrogen, respectively for twenty minutes. To obtain different oxygen concentrations, the solutions were mixed in different ratios. Measurements were performed with Fiber-Optic Oxygen Microsensor (Figure 4) in combination with the Microx TX3 device and the corresponding software. The obtained result was a linear decrease of the oxygen concentration. Specific activity was calculated from the slope and protein concentration. The results were processed in SigmaPlot.

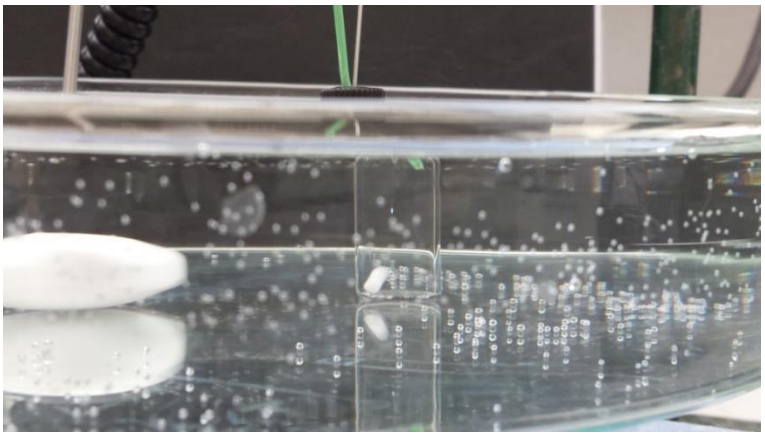

Figure 4. Fiber-Optic Oxygen Microsensor

3.4.2.1. Determining of the reaction mechanism for CDH N700S/N748G variant

To determine two substrate reaction mechanism, we kept the concentration of one substrate constant while we were measuring the activity of the variant at different concentrations of the other substrate. Measurements were performed with 0.5 mM, 1 mM and 3 mM cellobiose solutions as saturating substrate. Specific activity was determined with different O_2 concentrations. Analyses were performed as described above with exception of the cellobiose concentration.

3.4.3. Two phase reaction

Experimental setup is shown in Figure 5.

Figure 5. Reaction set-up

A 500 mL bioreactor with magnetic propeller agitator was chosen for the reaction. Reaction was carried out in a reaction system consisting of 200 mL aqueous and 40 mL organic phase. The reactor was immersed in a 30°C water bath, heated with a thermoplate. The temperature was monitored with a feedback control Pt100 Thermometer. As a cooling system, we used a Liebig condenser and a water bath at -20°C (F10, Julabo, Germany). The pH value was measured and kept constant at 7.0 with a BioController pH meter and its corresponding control unit. Since we expected that the pH is decreasing during the reaction, 1 M Na_2CO_3 was added automatically by the control unit into the system. Mixing was controlled with a magnetic stirrer (600 rpm) while we kept the oxygen concentration of 20 % constant with an Applikon flow console.

The aqueous phase was 300 mM cellobiose and the organic phase was pure ethylbenzene. The reaction was tested for different UPO/CDH ratios, in terms of activity: 5:1, 2:1, 1:1 respectively. The reaction began when 100 µL of UPO (104.2 U) and corresponding amount of CDH N700S/N748G variant were added to the reaction mixture (for 5:1 ratio we added 1.3 mL of the variant (16 U); for 2:1 we added 1.95 mL more and for 1:1 we added 3.3 mL more). Every three hours new amount of CDH was added. During the reaction the following parameters were monitored: UPO and CDH activity and the concentration of H_2O_2. Samples were also taken for the analysis of the phenylethanol and cellobiose concentration.

3.4.4. Stability of UPO and CDH N700S/N748G variant in different reaction conditions

Stability of enzymes was examined in different reaction conditions, as follows:

- 20 % oxygen, mixing speed 600 rpm
- 20 % oxygen, mixing speed 300 rpm
- 10 % oxygen, mixing speed 600 rpm

The reaction setup was identical as previously described. Water was used instead of cellobiose. Measurements were performed for three hours. We used 100 μL of UPO and 130 μL of CDH variant. Samples were analyzed for CDH and UPO activity.

4. RESULTS AND DISCUSSION

4.1. RESULTS AND DISCUSSION OF THE TRANSFORMATION PROCESS

4.1.1. *Aspergillus niger* transformation

Few days after the transformation was carried out, 16 transformed single colonies were subcultivated on new minimal medium plates. Out of 16 transformants found, twelve of them were likely to contain *Ao*CDH and the rest *Ct*CDH gene. Only one *Ct*CDH transformant continued to grow in the second generation (Figure 6.) The reason for such low efficiency could be that the desired gene was not integrated in the genome.

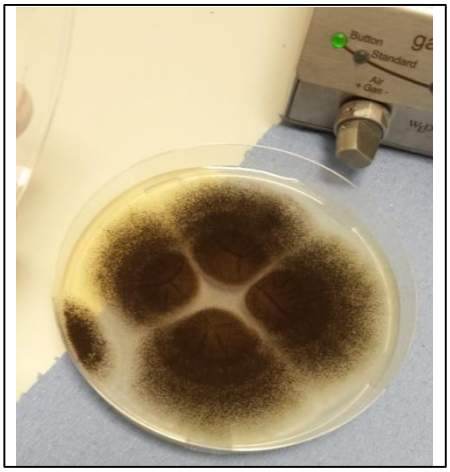

Figure 6. *A. niger Ct*CDH transformant

4.1.2. *Aspergillus niger Ct*CDH screening

*Ct*CDH transformed colony, for which we have assumed to have desired gene integrated in the genome, was subjected to screening. The screening was conducted as already described. The pH values can be seen in Appendix 2.

The crude extract samples from the 6th day were submitted to the DCIP activity assay and at the 11th day activity had reached its maximum (Table 9). Also for the samples from the last day of screening, the cyt *c* activity was measured. The results of these assays are

expressed as average values ± standard deviation and can be seen below (Table 9). The sequence analysis of the crude extract sample confirmed that produced enzyme was *Ct*CDH.

Due to high DCIP and cytochrome *c* activity of the crude extracts we decided to continue with the production. The crude extract from the last day was also subjected to the Bradford protein concentration assay.

Table 9. Activity of *Ct*CDH during the screening

Activity assay	**Day 6**	**Day 7**	**Day 8**	**Day 9**	**Day 10**	**Day 11**
DCIP [U mL^{-1}]	0.16 ± 0.06	0.23 ± 0.07	0.37 ± 0.07	0.50 ± 0.08	0.59 ± 0.13	0.62 ± 0.06
Cytochrome *c* [U mL^{-1}]	n.a.	n.a.	n.a.	n.a.	n.a.	0.17 ± 0.01

4.1.3. *Aspergillus niger Ct*CDH cultivation

For the production of *Ct*CDH we inoculated 15 non-baffled flask containing 250 mL of the same media used for the screening. The appearance of the flasks on the inoculation day can be seen on Figure 7. Figure 8 shows the appearance of flasks on the 9th day. The pH values, which had been monitored during the cultivation, can be found in Appendix 3.

Figure 7. Flask appearance on the inoculation day.

Figure 8. Appearance of flasks on the 9th day.

After 10 days, cells were harvested by filtration and discarded, whereas filtrates of the flasks were collected to give 2.4 L of the liquid crude extract. The crude extract was then purified as previously described. After each purification step, the samples were analyzed for the DCIP and the cyt c activity and the protein concentration was determined. In Table 10, the results of the analyses, as well as the calculated total protein mass and the specific activity, can be found.

Table 10. Results of the Bradford, the DCIP assay, the cytochrome c assay, the calculated total protein mass and the specific activity (results are shown as average value ± STD)

Sample	**Volume [mL]**	**Protein concentration [mg mL^{-1}]**	**Total protein [mg]**	**DCIP activity, pH=5.5 [U mL^{-1}]**	**DCIP Specific activity pH=5.5 [U mg^{-1}]**	**Cyt c activity, pH=7.0 [U mL^{-1}]**	**Cyt c Specific activity pH=7.0 [U mg^{-1}]**
Crude extract	2400	0.14 ± 0.01	331 ± 34	0.36 ± 0.02	2.58 ± 0.14	0.09 ± 0.01	0.64 ± 0.34
After HIC & vivaflow	40	0.15 ± 0.01	6.04 ± 0.29	1.44 ± 0.05	9.51 ± 0.30	0.33 ± 0.01	2.17 ± 0.02
After IEX:	2 fractions: a) 16 b) 20	2 fractions: a) 2.83 ± 0.13 b) 0.82 ± 0.01	2 fractions a) 45.3 ± 2.10 b) 16.4 ± 0.21	2 fractions: a) 50.2 ± 3.78 b) 12.1 ±0.19	2 fractions a) 17.7 ±1.33 b) 14.8 ± 0.23	2 fractions a) 14.9 ± 0.19 b) 2.07 ± 0.25	2 fractions a) 5.29 ± 0.07 b) 2.54 ± 0.31
Pure CDH	2 fraction a) 1.4 b) 1.0	2 fractions: a) 24.1 ± 0.36 b) 11.8 ± 0.46	2 fractions: a) 33.7 ± 0.49 b) 11.8 ± 0.46	2 fractions: a) 416 ± 3.51 b) 149 ± 5.25	2 fractions: a) 17.3 ± 0.15 b) 12.7 ± 0.44	2 fractions a) 99.6 ± 0.39 b) 38.8 ± 0.10	2 fractions: a) 4.13 ± 0.02 b) 3.27 ± 0.01

4.1.4. CDH characterization

Kinetics determination

To determine kinetic constants of *Ct*CDH in a reaction with cellobiose we measured the activity with different cellobiose concentrations. All samples and dilutions were prepared using the McIlvaine buffer. Dilutions of *Ct*CDH were 100 times for the DCIP assay and 700 times for the cyt *c* assay.

Although it has been reported that the enzyme follows the ping-pong reaction mechanism, the Michaelis-Menten model sufficiently well describes CDH's reaction kinetics and it is widely used in the literature for the calculation of kinetic parameters (Schultz, 2015) (Figures 9 and 10).

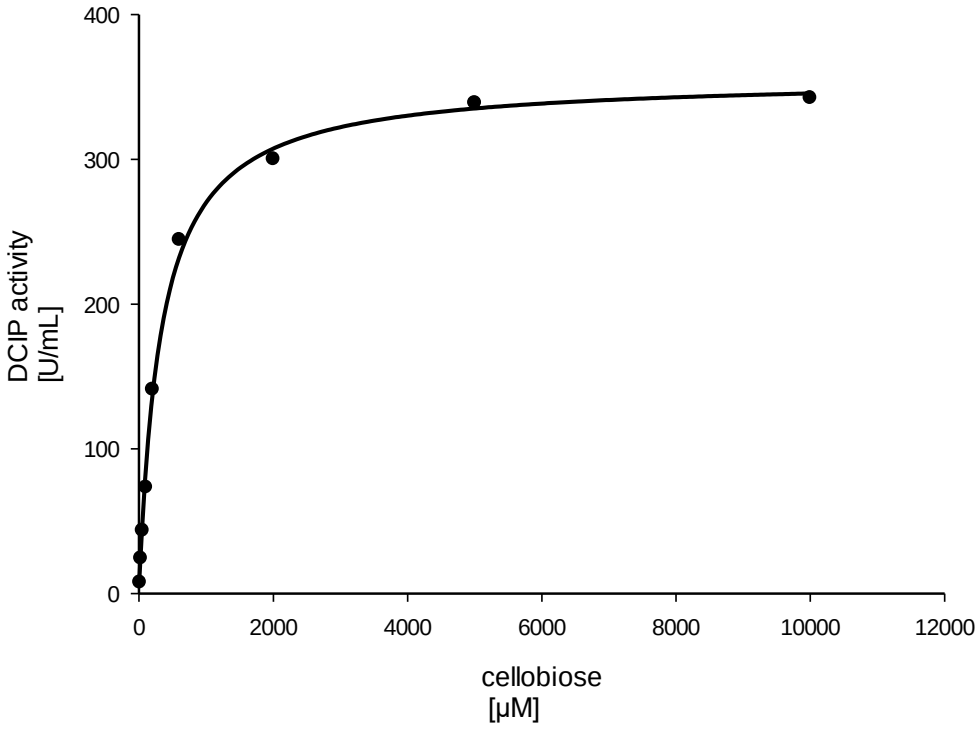

Figure 9. Activity of *Ct*CDH in the presence of different cellobiose concentrations measured with the DCIP assay at pH 5.5.

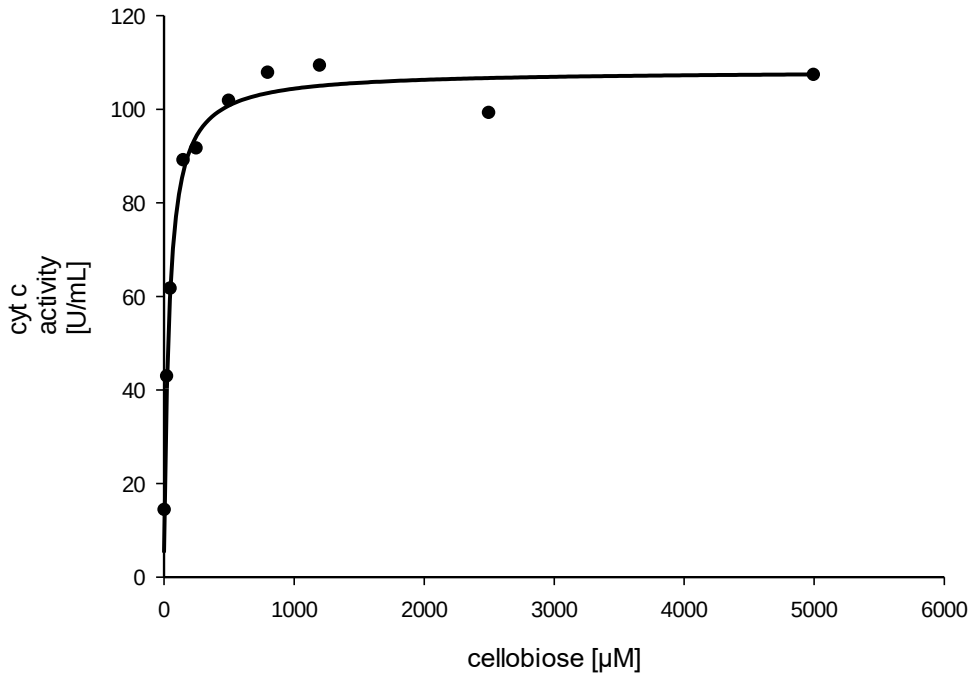

Figure 10. Activity of *Ct*CDH in the presence of different cellobiose concentrations measured with the cytochrome *c* assay at pH 7.5.

The V_{max} value determined with the DCIP assay is 356 U mL^{-1}, which corresponds to 14.8 U mg^{-1} whereas the K_M value is 320 µM. The measurements with the cyt *c* assay resulted in a V_{max} of 108 U mL^{-1} (13.3 U mg^{-1}) and a K_M of 37 µM.

4.2. RESULTS OF THE BIOCONVERSION PROCESS

4.2.1. Kinetic characterization of UPO

The activity of UPO in the presence of the different H_2O_2 concentrations can be seen below (Figures 11 and 12). The kinetic constant for inhibition couldn't be calculated from the obtained results. In this case, we can talk about the inactivation rather than the inhibition, but this is yet to be confirmed.

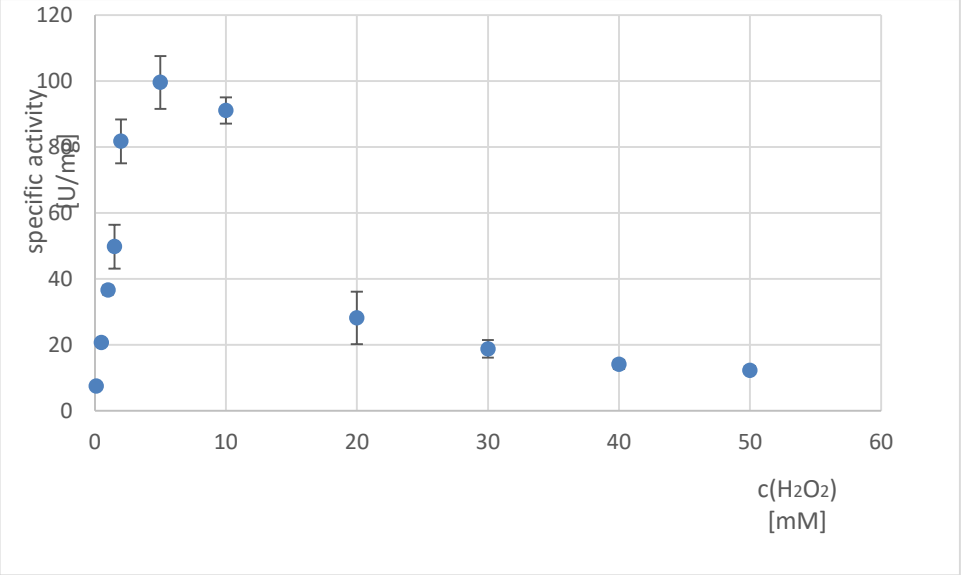

Figure 11. Activity of UPO in the presence of different H_2O_2 concentrations (termal denaturation of UPO).

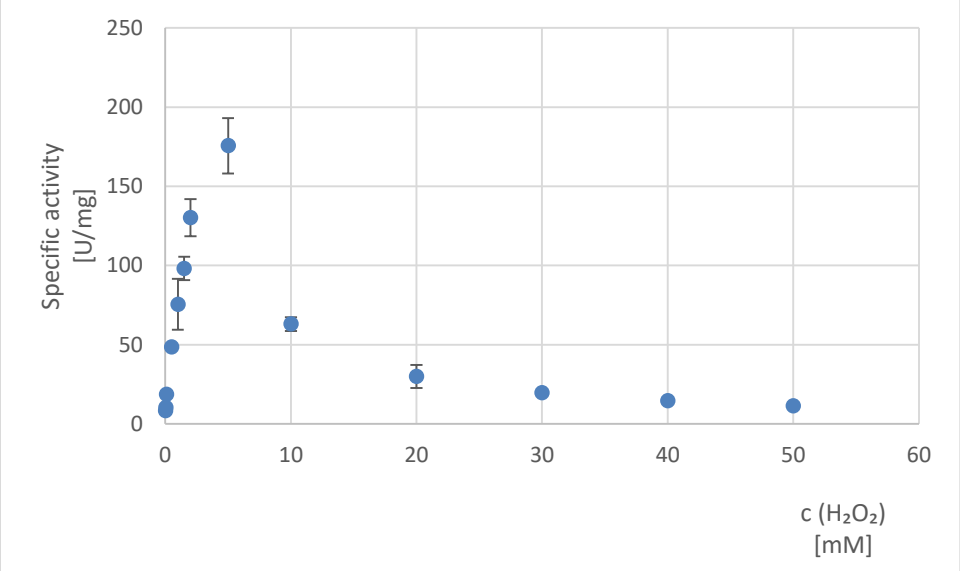

Figure 12. Activity of UPO in the presence of different H_2O_2 concentrations (acid denaturation of UPO).

Kinetics constants are calculated for the area with no inhibition. V_{max} and K_M values obtained from the thermal denaturation measurement are 252 U mg^{-1} and 2.15 mM, respectively. The acid denaturation measurement resulted with V_{max} 160 U mg^{-1} and K_M 2.82 mM.

4.2.2. Characterization of different CDH variants

In order to compare the oxygen activity of different CDH variants with activity of wild type DH and wild type CDH, we also analyzed wild type DH and wild type CDH. Since the electron transfer between the two CDH domains is strongly pH dependent, the measurements were also performed at pH 5.5. It is important to state that both of the reactions were very slow and results are just for comparison purposes. The molecular mass was calculated by EXPASy online tool for DH_{CDH} from position 208N on. The signal peptide was determined by SignalP4.1 and was removed before the calculation of the molecular mass for the whole CDH.

Table 11. Specific activity of flavocytoprotein CDH and dehydrogenase domain of CDH at 100% oxygen concentration and 10 mM cellobiose.

CDH type	**Specific activity pH=5.5 [kUmol^{-1}]**	**Specific activity pH=7 [kU mol^{-1}]**
wild-type DH_{CDH} MW = 66689.45 g mol^{-1}	4688	4508
wild-type CDH ME = 86623.31 g mol^{-1}	5475	3231

The specific activity at pH 7.0, depending of different oxygen concentrations as also as belonging K_M and V_{max} values, can be seen in the following figures.

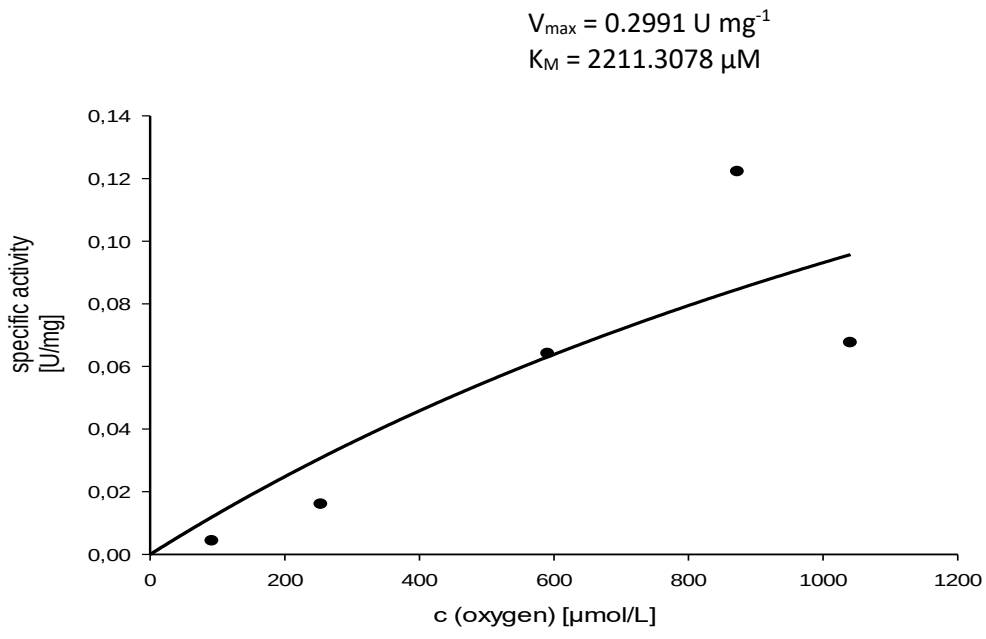

Figure 13. Wild-type DH

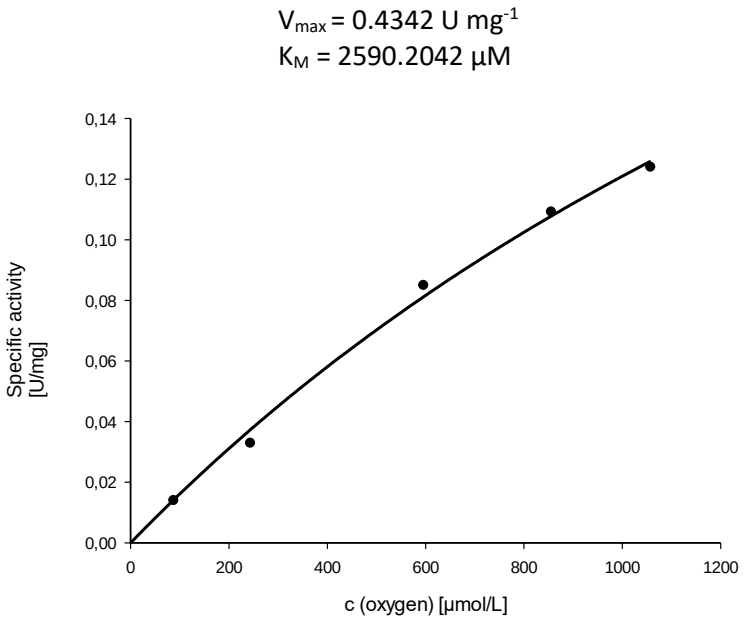

Figure 14. N700S

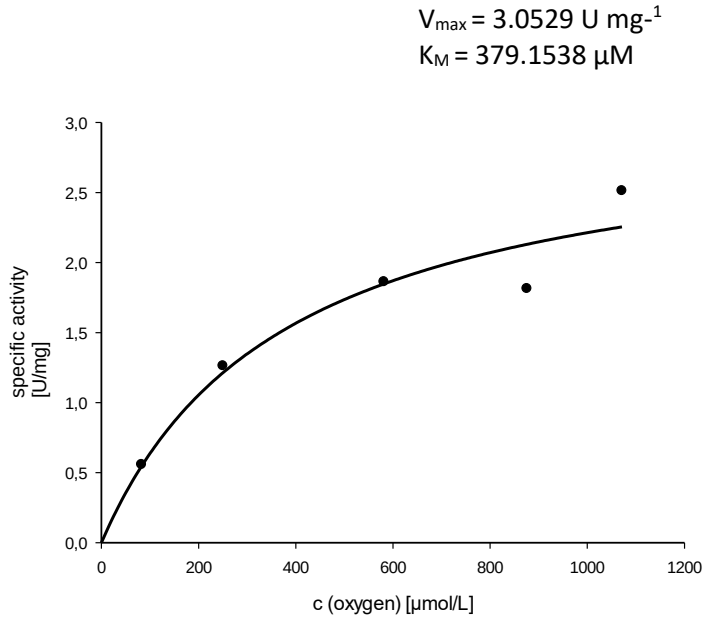

Figure 15. N748G

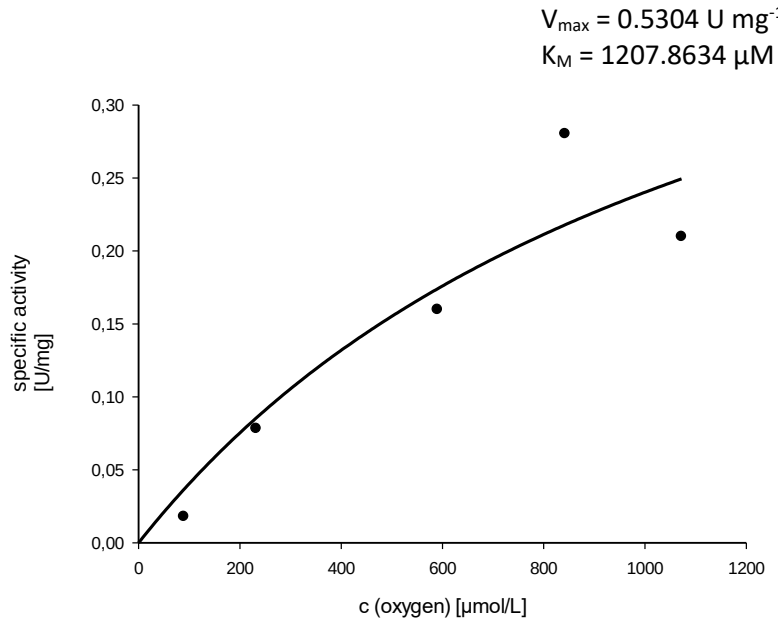

Figure 16. T750Q

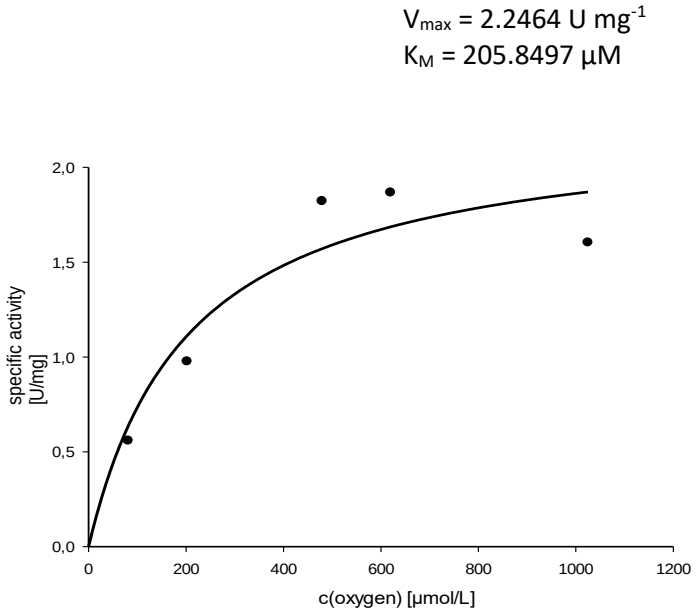

Figure 17. N700S/N748G/T750Q

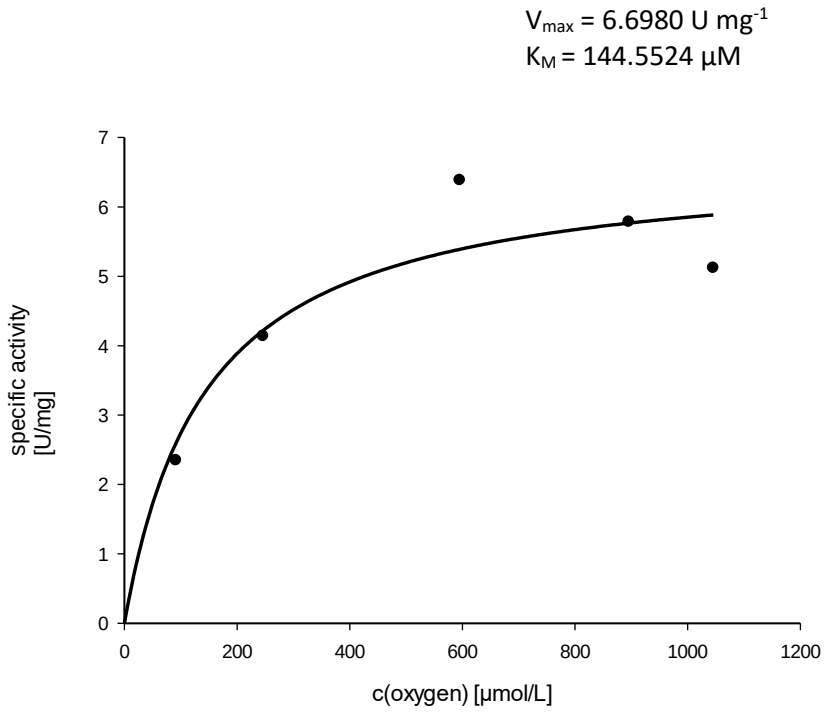

Figure 18. N748G/T750Q

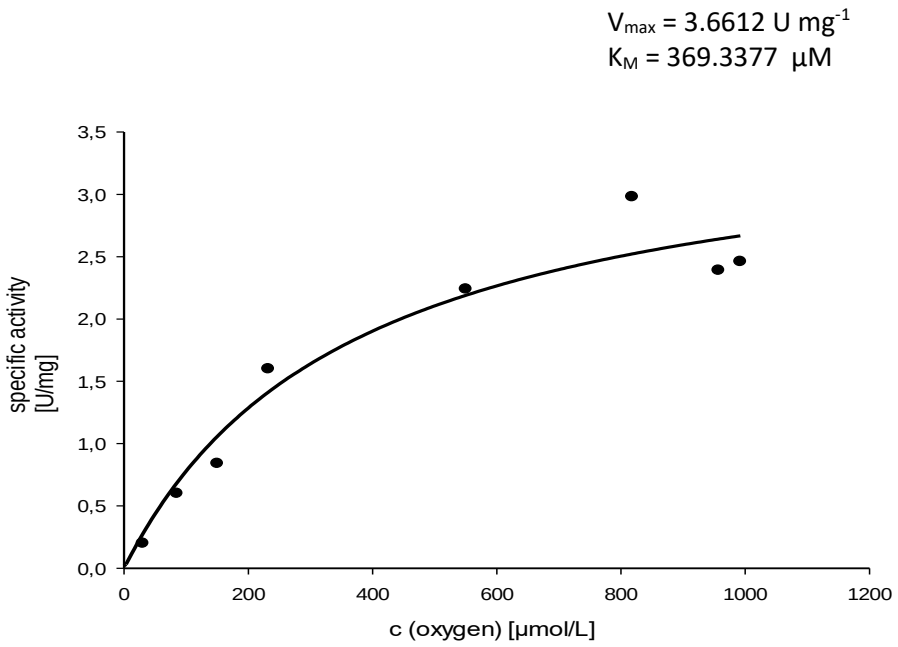

Figure 19. N700S/N748G

4.2.3. The two substrate reaction mechanism for CDH N700S/N748G variant

The results (Figure 20) showed that the reaction follows, so called, PING-PONG mechanism.

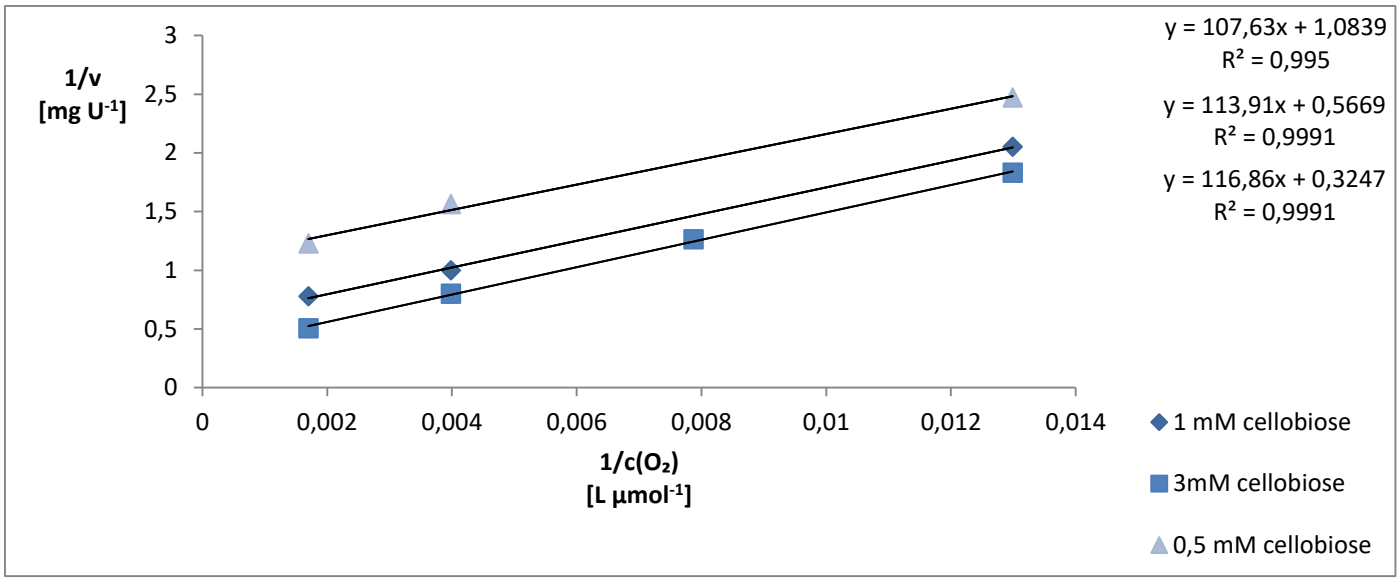

Figure 20. Two substrate reaction mechanism for CDH N700S/N748G variant

4.2.4. Specific activity of CDH N700S/N748G variant depending on different concentrations of cellobiose

The results obtained from the reaction mechanism analyses are used for determining the kinetic constants for cellobiose in presence of different oxygen concentrations. The specific activity depending of different cellobiose concentrations as also as the belonging K_M and V_{max} values, can be seen in the following figures.

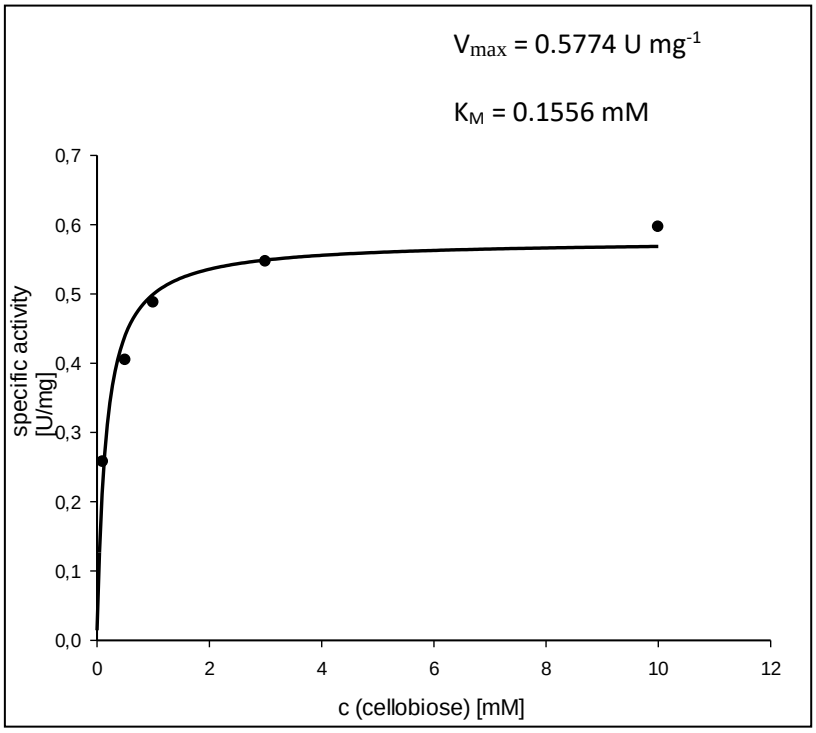

Figure 21. Specific activity depending on different concentrations of cellobiose with 5% oxygen.

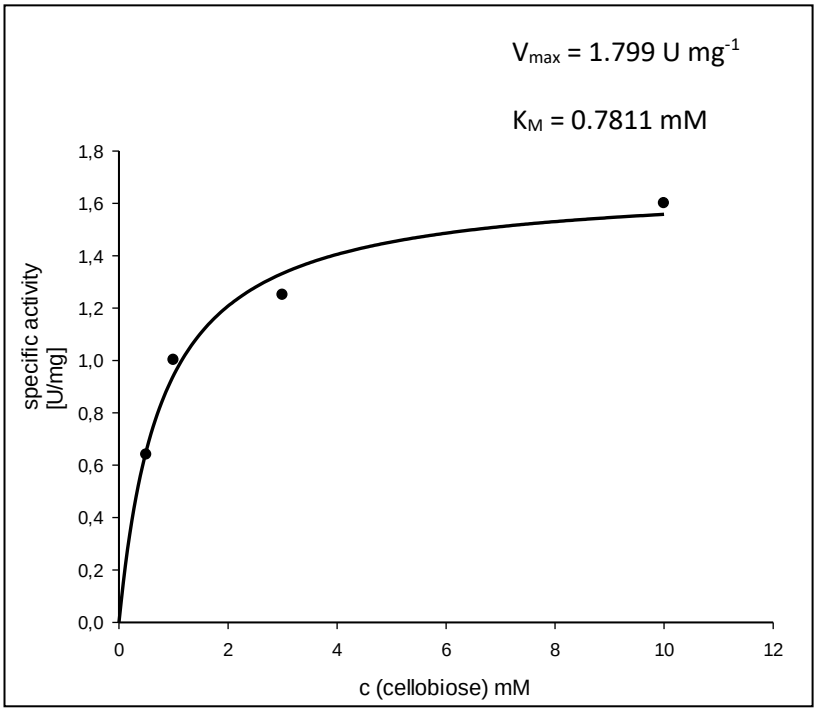

Figure 22. Specific activity depending on different concentrations of cellobiose with 20 % oxygen

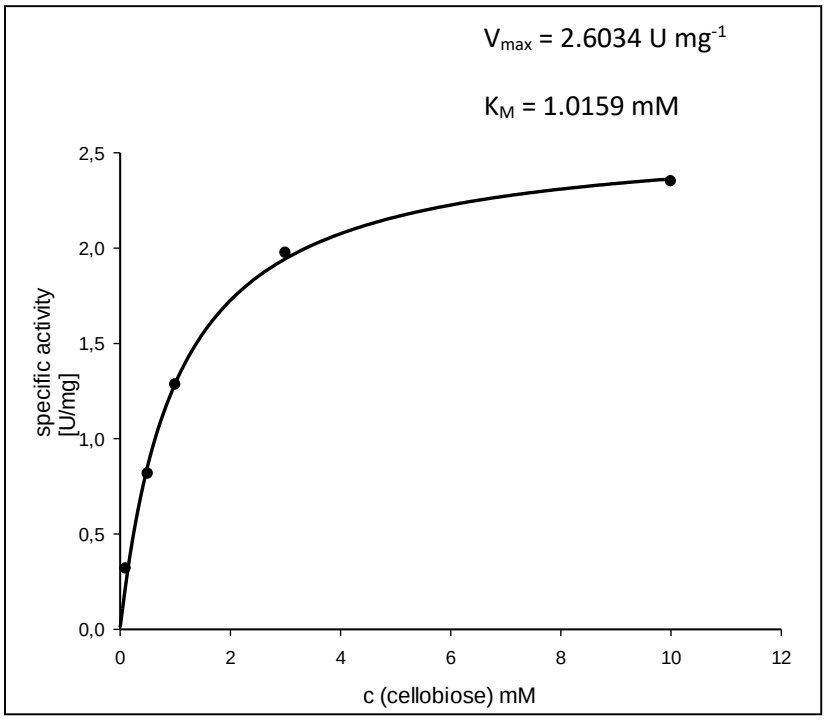

Figure 23. Specific activity depending on different concentrations of cellobiose with 50% oxygen.

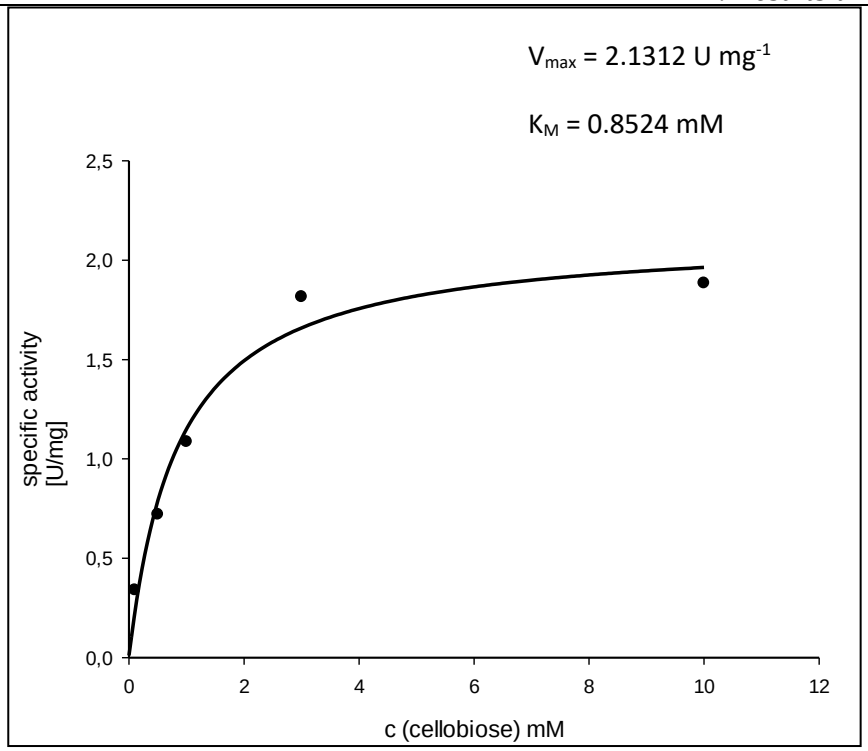

Figure 24. Specific activity depending on different concentrations of cellobiose with 100% oxygen.

4.2.5. Two-phase reaction

CDH or the DH domain of CDH can take part in two-phase bioconversion of ethylbenzene to phenylethanol together with enzyme unspecific peroxygenase (UPO). For this specific reaction it is important to use a CDH variant or, better its DH domain, with higher oxygen affinity, since as already stated, the reaction of wild type CDH with oxygen is very low. Bioconversion is conducted in two separated reactions which take place in aqueous phase while one substrate is in organic phase. In the first reaction cellobiose is oxidized to cellobionic acid wherein the FAD of DH is then reduced to $FADH_2$. In order to be reoxidized, $FADH_2$ donates two electrons to oxygen, which is than reduced to H_2O_2. H_2O_2 is then reduced by UPO in a reaction where ethylbenzene diffuse from organic to aqueous phase and gets oxidized to phenyethanol which diffuses back to the organic phase.

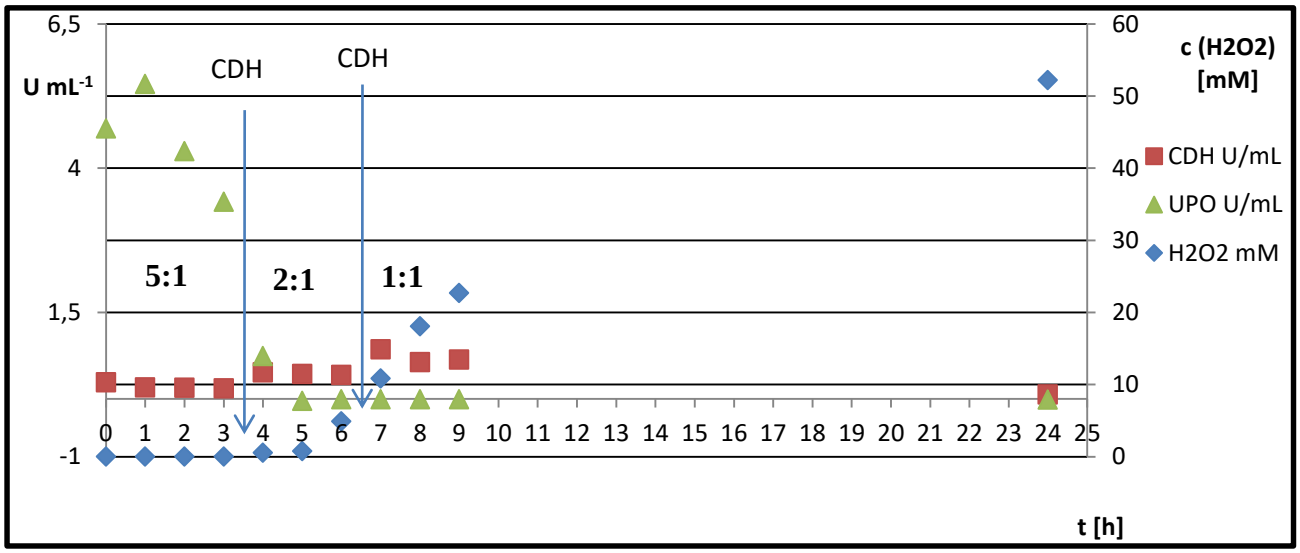

Figure 25. Activity of UPO and CDH, concentrations of H_2O_2 during the reaction.

During the reaction we noticed very fast inactivation of UPO. To determine which factors are crucial for enzyme stability, we tested the enzyme activity in different reaction conditions, as it can be seen in the next section.

4.2.6. Stability of UPO and CDH N700S/N748G variant in different reaction conditions.

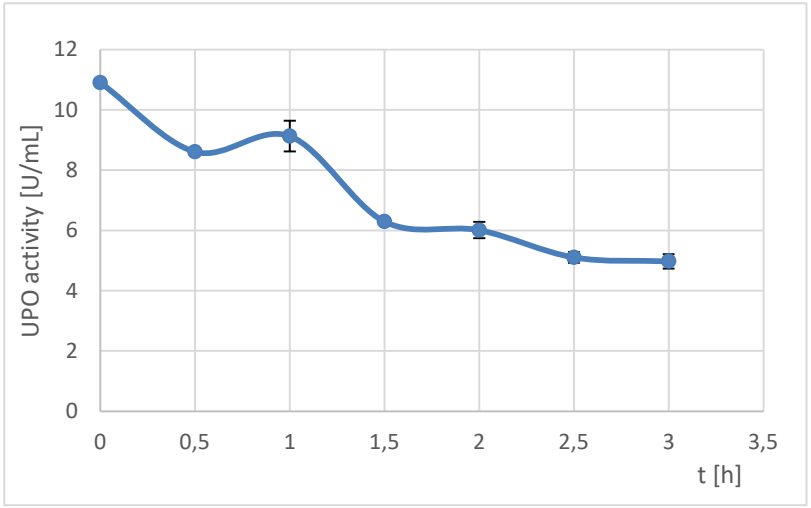

Figure 26. Stability of UPO at 20% O_2, 600 rpm.

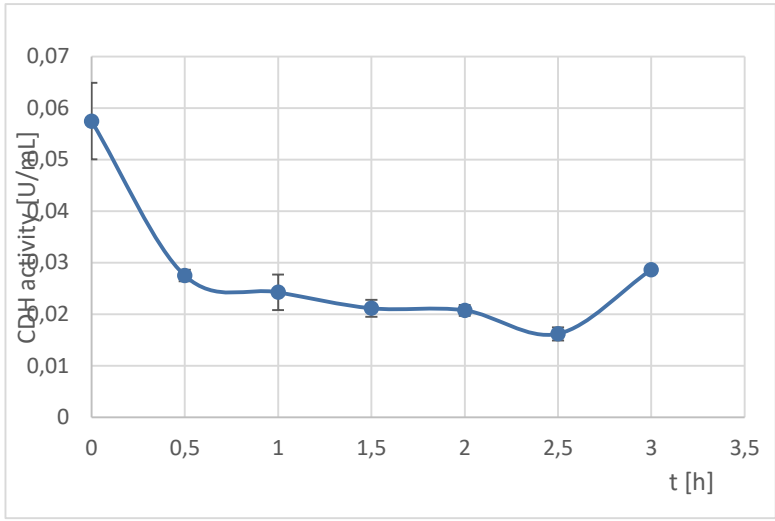

Figure 27. Stability of CDH N700S/N748G at 20% O_2, 600.

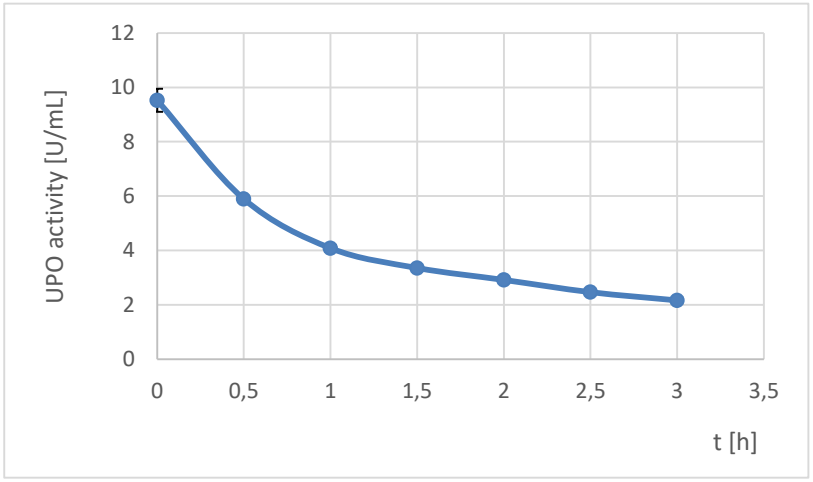

Figure 28. Stability of UPO at 20% O_2, 300 rpm.

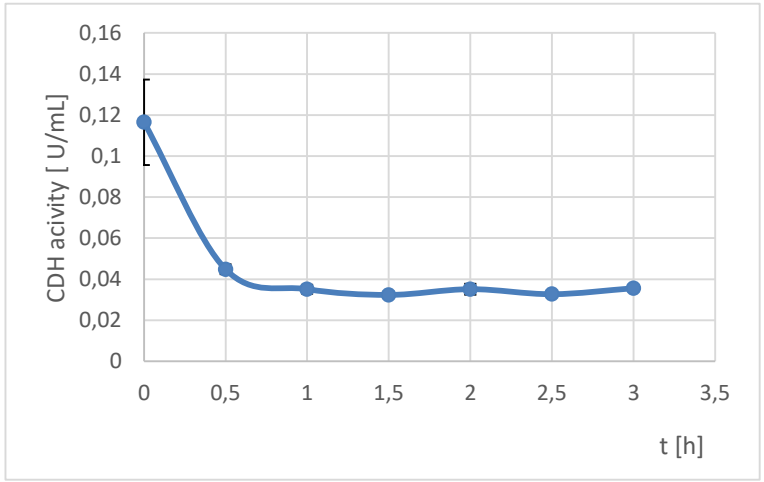

Figure 29. Stability of CDH N700S/N748G at 20% O_2, 300 rpm.

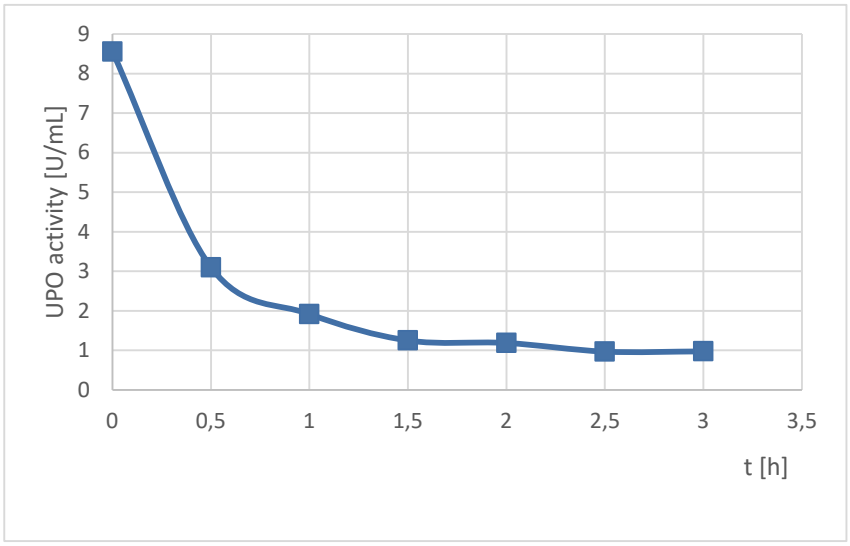

Figure 30. Stability of UPO at 10% O_2 and 300 rpm.

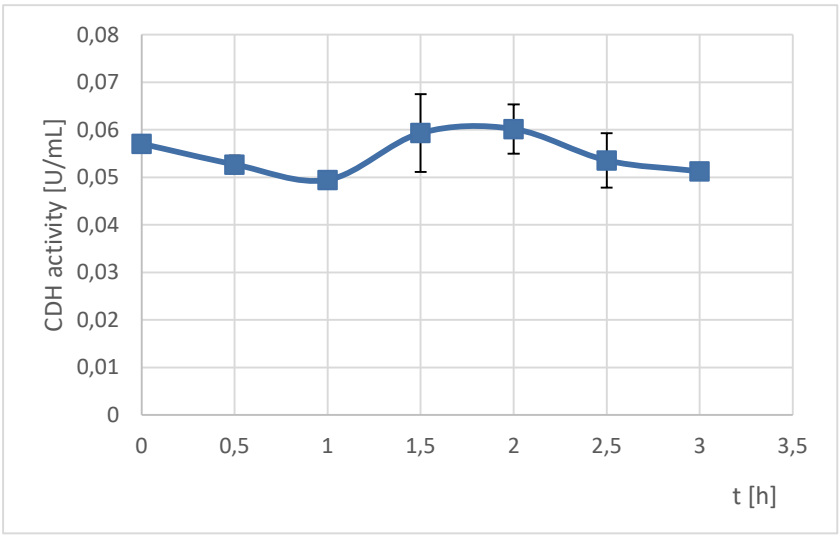

Figure 31. Stability of CDH N700S/N748G at 10% O_2 and 300 rpm.

4.3. DISCUSSION: TRANSFORMATION PROCESS

As the most abundant raw material on the Earth, lignocellulosic biomass has great potential as the main material in numerous bioprocesses (Nigam and Pandey, 2009; Philippoussis, 2009), but it is important to find the most efficient way to break down that kind of material to a less complexed structure. Lignocellulose-degrading enzymes, among which CDH, play a great role in that process. To avoid complexed and time consuming cultivation of the natural CDH producers, *cdh* genes are cloned in different, well-known expression hosts which enable fast, reliable and efficient enzyme production (Ludwig et al., 2013).

Due to genetic and evolutionary resemblance it can be speculated if and which fungal expression system is the best for the heterologous expression of CDH genes from white and brown rot, phytopathogenic and composting fungi. As expected, the recombinant production of holoenzyme in a bacterial expression system was reported as inefficient due its inability to perform posttranslational modifications (Harreither et al., 2012), while several attempts to express CDH in a yeast expression system were successful (Harreither et al., 2012; Ludwig et al., 2013). To examine the efficiency of another fungal expression system, the heterologous expression of the *cdh* gene from *Corynascus thermophilus* in *Aspergillus niger* was studied as well as the heterologous expression of the *cdh* class III gene from *Aspergillus oryzae.*

The project was carried out as, so called, cotransformation, where we had produced and isolated the selection marker and the desired gene separately in corresponding *Escherichia coli* strains. Few days after the transformation was carried out, 16 transformants were obtained. Out of 16 transformants found, twelve of them were likely to contain *Ao*CDH and the rest *Ct*CDH gene. Since only one *Ct*CDH transformant continued to grow in the second generation, that colony was chosen for the screening process. The reason for such low efficiency could be that the desired gene was not integrated in the genome. During the screening, high CDH activity was detected, in an average of 620 U L^{-1} (Table 9).

A picture of the SDS-PAGE gel with the crude extract sample after the screening can be found in Appendix 1. The band between 75 and 100 kDa is visible which corresponds to values reported in the literature (Ludwig et al., 2013). Furthermore, the band is not smeared indicating that the produced CDH is very pure.

Since the screening showed very good activity, we continued with the production in the same cultivation conditions to avoid consumption of nutrients for the growth instead of the enzyme production. *Aspergillus* grew in form of pellets. In every flask there were

approximately same-size pellets as every flask was cultivated with the same concentration of spore solution (Figures 7 and 8).

Interestingly, during the production process, enzyme activity measured by the DCIP-assay was half as high as during the screening (Appendix 4). The volumetric activity during the production was similar to the reported value (376 U L^{-1}) for recombinant production of *Ct*CDH in *Pichia pastoris* (Harreither et al., 2012; Ludwig et al., 2013), while it had been doubled during the screening process (Table 9). Reasons are left for speculation, since both, the screening and the production were carried out in the same conditions.

During the cultivation we noticed daily increase of the pH value resulting with the maximum value of 6.5 (Appendix 3). This was expected since it corresponds to the described natural conditions with pH values increasing from pH 5.0 to 7.5, as for example, in soil or compost (Harreither et al., 2011). This also suggests a more extended pH working range of the produced enzyme (Harreither et al., 2011).

The purification yields are moderate because only the purest fractions were pooled, which due to different color, were collected in two separated fractions (Table 10). We assumed that the one with darker color could contain the holoenzyme and the lighter one is probably an isolated domain. This can be proved with SDS electrophoresis, but unfortunately, due to lack of time, it has not been checked. As it can be seen in Table 10, the results obtained with the DCIP and the cyt *c* assays are different, in fact, the results of the DCIP activity assay are much higher (3-5 times) than those for cyt *c.* As already described, the difference between these two acceptors is that DCIP is reduced by dehydrogenase domain of CDH, while cyt *c* interacts only with the cytochrome domain of CDH. Interestingly, the calculated specific activity of the purified CDH at pH 5.5 for DCIP (Table 10) was similar to the one reported for the wild type *Ct*CDH obtained in a cellulose containing medium (17.8 U mg^{-1}) (Harreither et al, 2011). The specific activity for cyt *c* at pH 7.0 was as expected, 4 times lower. The obtained results are higher from those reported for *Ct*CDH expressed in *Pichia pastoris* (Harreither et al., 2012), for DCIP 4.2 times higher and for cyt *c* 1.45 times higher.

To determine kinetic constants of *Ct*CDH in a reaction with cellobiose we measured the activity with different cellobiose concentrations (Figure 9 and 10). The K_M value determined with the DCIP assay was approximately twice of value reported for wild type *Ct*CDH as well as the maximum specific activity (Harreither et al., 2012). In 2012, Harreither

et al. also reported the K_M values with cyt *c* as electron acceptor which were, comparing the reported values here, 4.6 times lower for both wild type *Ct*CDH and recombinant *Ct*CDH produced in *Pichia pastoris.* Furthermore, the obtained V_{max} (U mg^{-1}) values are similar for both assays.

4.4. DISCUSSION: BIOCONVERSION PROCESS

Regarding the kinetic characterization of UPO, we can speculate about the inactivation rather than the inhibition, but this is yet to be confirmed. The maximum velocity for the acid denaturation method is 1.57 fold lower than for the thermal inactivation. This is most probably due to the immediate stop of the reaction with acid compared to the slow thermal inactivation. The K_M value is not as affected as the V_{max} since there is no such big difference between the values for the described ways of inactivation (Figures 11 and 12).

The reactions of cellobiose oxidation in presence of 100 % oxygen catalyzed separately with the flavocytoprotein CDH and the dehydrogenase domain of CDH were very slow, so the obtained results are just for comparison purposes (Table 11). The isolated dehydrogenase domain shows the same activity at both its pH optimum 7 and pH 5.5. For the wild type CDH there is a difference, at pH 7 the activity is almost half of the activity at 5.5, which confirms that electron transfer between two CDH domains is strongly pH dependent and it has a maximum at 5.5 (Harreither et al.2011). Further measurements are needed to confirm the data.

Different publications have reported CDH's low tendency for oxygen reduction (Kracher et al., 2016), so the final outcome of the introduced mutations described in material and methods was a higher oxygen affinity. The influence of different mutations of CDH variants is noticeable if we compare kinetic data for each mutation (Figures 13-19). If we compare V_{max} and K_M values of the wild type isolated domain with values obtained from the single variant N700S, we can see that there is not significant improvement in oxygen affinity since there is no considerable difference between K_M values. Also the V_{max} value of the variant is slightly higher than wild type DH. Opposite to the previous single mutant, the mutation in the variant N748G resulted in higher O_2 affinity since the K_M value of this variant is almost 6 times lower compared to value of the wild type isolated domain. The V_{max} value is 10 times higher than the wild type DH. The third single variant T750Q also shows improvement of kinetic data compared to the wild type DH, but not as good as variant

N748G. The double mutations in variant N700S/N748G resulted in similar kinetic data for single variant N748G indicating that replacing asparagine with serine at 700 th residue (N700S), does not have significant effect on oxygen affinity. The mutations in variant N748G/ T750Q were the most efficient, since the K_M value was around 15 times lower than the K_M for the wild type DH. The investigation of this variant also resulted in the highest V_{max} value. The obtained kinetic data from reactions with the triple variant were in between the results from the double variants. From this we can assume that the N700S variant does not have a positive impact on the oxygen affinity, whether alone or combined with other mutation.

Although the variant N700S/N748G is not the variant with the highest oxygen affinity, it was chosen for two substrate reaction mechanism determinations, because of available quantities. The results showed that this specific reaction, with cellobiose and oxygen as substrates follows the, so called, PING-PONG reaction mechanism (Figure 20). This corresponds to literature since this reaction mechanism was reported for all oxidoreductases (Schulz, 2015). Cellobiose binds first and is oxidized to cellobiono-1.5-lactone while FAD is reduced to $FADH_2$. The product is then released and undergoes spontaneous hydrolysis to cellobionic acid. The second substrate, (oxygen) is then bound, after which FAD is reoxidized by oxygen. Oxygen is reduced to hydrogen peroxide and released from the enzyme. Although we can confirm that the reaction mechanism does not follow the Michaelis-Menten model, nevertheless we used it for calculation of kinetic parameters as it is done in most publications (Schulz, 2015) (Figures 13-24).

The kinetic constants of the variant N700S/N748G for cellobiose are obtained by combining the results from the reaction mechanism analyses (Figures 21-24). With increasing oxygen concentration changes in K_M and V_{max} values are visible. There is a trend, up to 50 % of oxygen concentration, the K_M value increase as well as the V_{max}. This can be explained by the following: the increment of the K_M does not mean lower cellobiose affinity, but with higher oxygen concentration the cofactor is faster reoxidized. This means with faster reoxidation of cofactor, that CDH has "less time" to bind cellulose and that is reflected in a higher K_M value. V_{max} is higher with higher oxygen concentrations, as expected. K_M and V_{max} values with 100 % oxygen do not fit in described "half reaction theory". Reason could be connected to inhibition of reaction in presence of oxygen concentration higher than 80 % (Figure 19).

The same variant was used in an enzyme cascade reaction within a two phase reaction setup (Figure 25). The reaction conducted in this way was designed to be more environmental friendly since it can be conducted under mild reaction conditions. Moreover, if air is used instead of the pure oxygen, the process also can be cost friendly. During the reaction we have already noticed significant inactivation of UPO in first three hours. CDH kept its activity as new quantities were added every three hours, but it is hard to say what percentage of CDH added at the beginning saved its stability and activity. Stated can be consequence of combination of a few factors such as presence of organic phase and mixing rate, oxygen concentration and temperature. Conducting the reaction in different reaction conditions should have resulted in an answer to the question which factors are crucial for enzyme stability. Both CDH and UPO showed no good stability in given conditions (Figures 26-31). Apparently, the oxygen concentration has a bigger effect on CDH stability than the mixing rate, since there is high loss of activity in first half an hour with higher examined oxygen concentration while with lower concentration activity of CDH kept constant. In contrast to that, UPO has significant loss of activity with lower oxygen concentrations, although in general we can say that in given conditions UPO is not stable. Further measurements need to be done since these changes in the reaction-setup did not improve the stability of UPO.

In 2010 Ludwig et al. reported several cases in which the stability of CDH was a problem. Among these are: denaturation by gas/liquid interference in air-mixed bioreactors and systems with higher concentration of hydrogen peroxide. The loss of activity was also reported as result of the adsorption of CDH to natural and artificial polymers, such as cellulosic and glass surfaces. Ultimately, further improvement must be achieved regarding enzyme stability, for example, with genetic manipulations or better reaction design.

5. CONCLUSIONS

Based on the obtained results in this thesis, the following can be concluded:

1. The heterologous expression of cellobiose dehydrogenase from *Corynascus thermophilus* can be successfully done in *Aspergillus niger* as expression host, while for recombinant expression of class III cellobiose dehydrogenase from *Aspergillus oryzae* further studies must be done
2. During the production, *Ct*CDH produced in *Aspergillus niger* showed similar characteristics to wild-type *Ct*CDH. It also had higher specific activity comparing to *Ct*CDH produced in *Pichia pastoris*. Produced enzyme showed higher K_M value for cellobiose comparing to both, wild type *Ct*CDH and *Ct*CDH produced in *Pichia pastoris*.
3. Higher oxygen affinity of the dehydrogenase domain of CDH can be successfully obtained with genetic engineering; mutations in variant N748G/ T750Q seem to be the most efficient.
4. CDH catalyzes two-substrate reaction that follows a ping-pong reaction mechanism.
5. CDH variants (produced by recombination) can be applied in organic chemistry. The limiting factor in the two-phase bioconversion process seems to be the enzyme's stability on which further research must focus.

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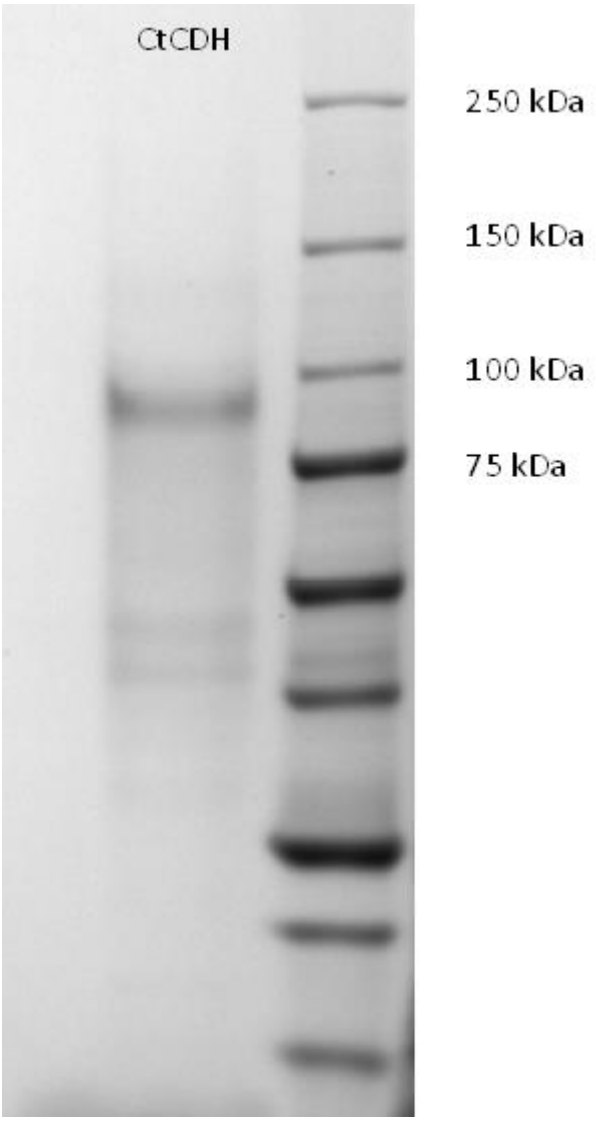
7. APPENDICES



Appendix 1. SDS-PAGE showing the molecular mass standard (lane 2) and *Ct*CDH (lane 1).

Mini-PROTEAN TGX Precast Gels (Bio-Rad, USA), Mini PROTEAN Tetra Cell (Bio-Rad, USA), and the PowerPac HC (Bio-Rad, USA) power supply were used for the electrophoresis. All procedures were done according to the manufacturer's (Bio-Rad Laboratories) recommendations.

In the first well 15 µL of the denatured sample was loaded while in the second one 15 µL of the unstained Precision Plus Protein Standard (Bio-Rad, USA) was loaded. The

electrophoresis was run at 120 V for about an hour. Protein bands were visualized by staining with QC Colloidal Coomassie (Bio-Rad, USA) and washed using distilled water.

Appendix 2. The change of the pH value during the screening.

		Day3		**Day 4**		**Day 5**		**Day 6**		**Day 7**		**Day 8**		**Day 9**		**Day 10**	
Flask	Starting pH value	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
I	5.5	5.9	5.0	5.0	5.0	5.3	5.3	6.2	5.3	5.9	5.3	6.3	5.3	6.5	5.6	6.5	5.6
II	5.5	5.9	5.0	5.3	5.3	5.9	5.3	6.2	5.3	5.9	5.3	5.9	5.3	6.8	5.6	6.8	5.6
III	5.5	5.9	5.0	5.3	5.3	6.2	5.3	5.3	5.3	5.9	6.2	6.5	5.3	6.5	5.6	6.5	5.6

Appendix 3. The change of the pH value during the cultivation.

		Day3		**Day 4**		**Day 5**		**Day 6**		**Day 7**		**Day 8**		**Day 9**		**Day 10**	
Flask	Starting pH value	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
I	5.5	6.2	5.3	5.6	5.6	5.9	5.6	5.6	5.6	5.9	5.3	5.9	5.3	5.9	5.3	5.9	5.3
II	5.5	6.2	5.3	5.6	5.6	5.9	5.6	5.6	5.6	5.6	5.3	5.9	5.3	6.2	5.6	6.2	5.6
III	5.5	6.2	5.3	5.6	5.6	5.9	5.6	5.6	5.6	5.9	5.3	5.9	5.3	6.2	5.6	6.5	5.6

IV	5.5	6.5	5.6	5.9	5.6	5.9	5.6	5.6	5.6	5.9	5.3	5.9	5.3	6.2	5.6	6.5	5.6
V	5.5	6.2	5.3	5.9	5.6	5.9	5.6	5.6	5.6	5.9	5.3	5.9	5.3	6.2	5.6	6.5	5.6
VI	5.5	6.2	5.3	5.9	5.6	5.9	5.6	5.6	5.6	5.6	5.3	5.6	5.3	6.2	5.6	6.5	5.6
VII	5.5	6.2	5.3	5.9	5.6	5.9	5.6	5.6	5.6	5.9	5.3	5.9	5.3	6.2	5.6	6.5	5.6
VIII	5.5	5.9	5.3	5.9	5.6	5.9	5.6	5.6	5.6	5.9	5.3	5.9	5.3	5.9	5.3	6.5	5.6
IX	5.5	6.2	5.3	5.9	5.6	5.9	5.6	5.6	5.6	5.9	5.3	5.9	5.3	6.2	5.6	6.5	5.6
X	5.5	5.9	5.3	5.9	5.6	5.9	5.6	5.6	5.6	5.9	5.3	5.9	5.3	6.2	5.6	6.5	5.6
XI	5.5	6.2	5.3	5.9	5.6	5.9	5.6	5.6	5.6	5.9	5.3	5.9	5.3	5.9	5.3	6.8	5.6
XII	5.5	6.2	5.3	5.9	5.6	5.9	5.6	5.6	5.6	5.9	5.3	5.9	5.3	6.2	5.6	6.5	5.6
XIII	5.5	6.2	5.3	5.9	5.6	5.9	5.6	5.6	5.6	5.9	5.3	5.9	5.3	6.2	5.9	6.5	5.6
XIV	5.5	6.2	5.3	5.9	5.6	5.9	5.6	5.6	5.6	5.6	5.3	5.9	5.3	6.2	5.6	6.5	5.6
XV	5.5	6.2	5.3	5.9	5.6	5.9	5.6	5.6	5.6	5.9	5.3	5.9	5.3	6.2	5.6	6.5	5.6

Appendix 4. The change of the activity during the cultivation (average value ± STD).

	Day 7	**Day 8**	**Day 9**	**Day 10**
Flask	**Activity [U mL^{-1}]**	**Activity [U mL^{-1}]**	**Activity [U mL^{-1}]**	**Activity [U mL^{-1}]**
I	0.19 ± 0.006	0.15 ± 0.022	0.17 ± 0.001	0.22 ± 0.014
II	0.24 ± 0.020	0.31 ± 0.019	0.31 ± 0.020	0.33 ± 0.008
III	0.29 ± 0.006	0.31 ± 0.030	0.33 ± 0.020	0.38 ± 0.013
IV	0.31 ± 0.038	0.32 ± 0.002	0.33 ± 0.002	0.36 ± 0.007
V	0.29 ± 0,011	0.34 ± 0.062	0.33 ± 0.009	0.48 ± 0.001
VI	0.16 ± 0.007	0.19 ± 0.021	0.18 ± 0.037	0.35 ± 0.002
VII	0.23 ± 0.001	0.27 ± 0.060	0.31 ± 0.012	0.29 ± 0.030
VIII	0.23 ± 0.035	0.26 ± 0.001	0.25 ± 0.003	0.28 ± 0.007
IX	0.13 ± 0.004	0.14 ± 0.020	0.20 ± 0.057	0.21 ± 0.017
X	0.27 ± 0.010	0.35 ± 0.080	0.45 ± 0.039	0.45 ± 0.044
XI	0.15 ± 0.006	0.17 ± 0.020	0.21 ± 0.072	0.26 ± 0.056

XII	0.28 ± 0.004	0.35 ± 0.002	0.37 ± 0.037	0.43 ± 0.016
XIII	0.22 ± 0.004	0.28 ± 0.015	0.39 ± 0.065	0.30 ± 0.005
XIV	0.24 ± 0.004	0.28 ± 0.030	0.31 ± 0.065	0.31 ± 0.055
XV	0.35 ± 0.008	0.38 ± 0.060	0.43 ± 0.015	0.42 ± 0.062